TAMPERE UNIVERSITY OF TECHNOLOGY

ELENA LOKHMAN

DEVELOPMENT OF SYSTEM FOR VISUALIZING NATURAL TRANSFORMATION IN REAL TIME

Master of Science Thesis

Examiners: Adjunct professor
Ville Santala and
Professor Matti Karp
Examiners and topic approved in the Faculty of Science and Environmental Engineering council meeting on 9th October, 2013

ABSTRACT

TAMPERE UNIVERSITY OF TECHNOLOGY
Master's Degree Programme in Biotechnology
LOKHMAN, ELENA: Development of system for visualizing natural transformation in real time
Master of Science Thesis, 68 pages, 3 Appendix pages
June 2014
Major: Biotechnology
Examiners: Professor Matti Karp and Adjunct professor Ville Santala
Keywords: Horizontal gene transfer, natural transformation, luminescence, *Acinetobacter*

Horizontal gene transfer (HGT) is a common event in nature and one of the major sources of novelties and diversity in microorganisms. Natural transformation is one of the HGT options and is the state during which bacteria is capable of taking free genetic material from the environment and recombining it in own chromosome.

Conventional laboratory method for observation of natural transformation and HGT based on growth selection has substantial limitations, such as underestimation of real transformation frequency, use of antibiotic resistance markers and general laboriousness. Therefore, some attempts to visualize HGT directly have been made with employment of fluorescent proteins and luminescence production as an alternative to conventional markers.

This study has made an attempt to create the new system for tracking the natural transformation in the real time with use of luminescence production as a fast measure of transformation extent. *Acinetobacter baylyi* ADP1 known for its natural competence and easy handling, full genome sequence of which is available, was chosen as a model organism for these manipulations.

The developed system was tested with several combinations of knock-out genes and antibiotic resistance markers, demonstrating the significant divergence in function of each particular combination and revealing additional factors influencing the natural transformation. Therefore, the initial goal to correlate the light production with the amount of positive transformants quantitatively and calibrate the system for more or less universal application proved to be difficult.

Nevertheless, the developed system was successfully applied for trial experiments to study the HGT between *A. baylyi* ADP1 and *Escherichia coli*. Additionally, it demonstrated the promising results in observing and confirming two-component simultaneous transformation in real time with potential to be further developed for refining of this experiment. Furthermore, some modifications to the system have been suggested to improve its functioning and further application ideas were proposed.

PREFACE

This thesis is based on the experiments conducted during February 2012 to May 2013 at the Department of Chemistry and Bioengineering in Tampere University of Technology, Finland.

I would like to sincerely thank my supervisor, Adjunct professor Ville Santala, for his help and guidance during the process, for creative approach to experiments and discussions, which turned this work to curiosity-driven adventure, and for his kind support in difficult moments.

In addition, I am very grateful to Professor Matti Karp and his research group for the opportunity to take part in regular group meetings and their help and advices given during these discussions.

Also I would like to give special thanks to my friends and family for their support and encouragement, and especially to my husband, Alexander Lokhman, for his assistance in automation of data processing in this study, countenance and endless patience.

April 2014, UK

ELENA LOKHMAN

CONTENTS

TERMS AND DEFINITIONS

ACP	Acyl-carrier protein
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CAM	Chloramphenicol
CLSM	Confocal laser scanning microscopy
dCAM	Damaged chloramphenicol
DIR	Double illegitimate recombination
dsDNA	Double stranded deoxyribonucleic acid
FMN	Flavin mononucleotide
GFP	Green fluorescent protein
GTAs	Gene transfer agents
HFDIR	Homology-facilitated double illegitimate recombination
HFIR	Homology-facilitated illegitimate recombination
HGT	Horizontal gene transfer
HR-SEM	High resolution scanning electron microscope
IR	Illegitimate recombination
KAN	Kanamycin
5'- LR	5'- non-coding leader region
MCS	Multiple cloning site
mRNA	Messenger ribonucleic acid
NADP	Nicotinamide adenine dinucleotide phosphate
rRNA	Ribosomal ribonucleic acid
SSC	Side scatter
ssDNA	Single stranded deoxyribonucleic acid
Tfp	Type IV pili
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 INTRODUCTION

Many genes-sharing events, such as spread of antibiotic resistance and pathogenicity determinants can be attributed to horizontal gene transfer (HGT). Natural transformation is one of the HGT options which does not involve any mobile genetic elements but relies on the development of natural competence state, which cell enters due to the specific intrinsic mechanism. This makes natural transformation distinct from the other HGT modes. Natural competence is the state during which bacteria is capable of taking free genetic material from the environment and recombining it in own chromosome or recircularizing the plasmid. Many bacterial species are known to be naturally competent under certain conditions. (Seitz & Blokesch 2013)

In common, natural transformation comprises four principal steps: stimulation of cell to enter the competence, binding of free DNA from environment, DNA traverse through the outer and inner membranes and its recombination with the corresponding homologous region in chromosome or plasmid regeneration (Gerischer 2008). However, the conditions contributing to the development of competence as well as mechanisms allowing natural transformation vary significantly between bacterial species. In addition to that, some microorganisms like *Helicobacter pylory* are constantly competent and do not require initiation. (Seitz & Blokesch 2013)

Many studies have been devoted to investigations of natural transformation process in different organisms, regulation of competence and exploration of gene transfer between particular species. It has been demonstrated that gene transfer is the event shared not only by bacteria but also can happen within and between all phylogenetic domains (Rest & Mindell 2003; Andersson et al. 2003; Gophna et al. 2004; Watkins & Gray 2006; Guljamow et al. 2007; Nedelcu et al. 2008, cited in Boto 2010). Therefore, the importance of HGT and natural transformations in evolution has become widely recognized during the last decades. In this connection, studies aimed to observe the HGT in laboratory and environment received the high attention in order to promote better understanding of the scale of this process in nature.

Conventional laboratory method for observation of HGT based on transformation and subsequent selection has substantial limitations. These include underestimation of the number of resulting transformants due to the loss of not culturable or not readily culturable bacterial fractions, overall laboriousness and significant time-spending which is required to get the data, and, as a consequence, inconvenience of this method for tracking the process in dynamic. Therefore, some attempts to visualize HGT directly have been made with employment of such widely used in biotechnology markers as fluorescent proteins and luminescence production. These experiments already now allowed to visualize gene transfer, for example, between plants and bacteria, clearly determine spatial and temporal characteristics of the process, showing the dimensional “hot spots” and avoiding the problem of sample depletion during experiments. Most of studies uti-

lizing fluorescence and all using luminescence, however, function according to general scheme, in which the reporter gene is repressed in the donor and escapes the repression once transferred to the recipient. In addition to that, to my best knowledge, there are only two studies applying the luminescence towards exploration of HGT and only conjugal mechanism in particular, while luminescence production has several advantages over fluorescent reporter genes.

Therefore, in this study I made the attempt to develop the new system for visualization of particularly natural transformation and HGT by means of luminescence production and detection, which is recognized to be more sensitive method in comparison with detection of fluorescence. *Acinetobacter baylyi* ADP1 known for its natural competence and easy handling, full genome sequence of which is available, was chosen as a model organism for these manipulations. Approach used in this thesis aimed to avoid the earlier referred general repression scheme but to allow the identification of transformants based on consequential recombination of cassettes containing different genes of *lux*-operon into *A. baylyi* ADP1 genome. Recombination of particular combination of cassettes within genome of one cell was supposed to lead to gradual collection of all the genes required for production of luminescence with addition of luciferase substrate or without. This was aimed to provide the tool not only for visualization of particular gene acquisition event but also for a bundle of events in dynamic in real time.

Moreover, all the genetic constructs comprising the system were supposed to have an advantage of being universal for any knock-out target, that is, produce comparable amount of luminescence, which had to be correlated with the number of positive transformants. For that, each construct was supposed to be “calibrated” with use of conventional plating method on a basis of harbored within each cassette antibiotic resistance. As only two resistance markers were available for work with *A. baylyi* ADP1, while lux-operon was initially supposed to be split between maximum three constructs, the luminescence was suggested as the third marker for identification of positive transformants on plates.

Finally, the system was planned to be tested on *A. baylyi* ADP1 as a model naturally competent microorganism. In addition to that, the experiments, which would apply the designed system to examination of the HGT possibility between on *A. baylyi* ADP1 and *Escherichia coli* were scheduled.

2 Gene transfer between different species and importance of horizontal gene transfer in evolution

Bacteria can easily adapt to the fast changing environmental conditions not only through cellular alterations and mutations but also through the horizontal spread of genetic information between cells. Experiments and phylogenomic studies demonstrated that HGT is a common event in nature and the process of genes acquisition and their spread to further generations is fast (Fig. 1.1). From the evolutional point of view, HGT is one of the major sources of novelties and diversity. It introduces new genes and corresponding functions to the recipient and influence natural selection. (Boto 2010; Popa & Dagan 2011)

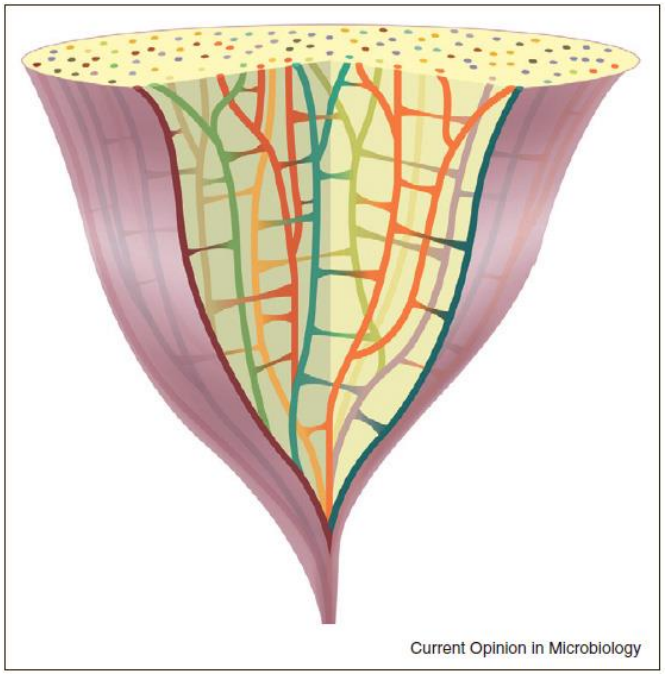

Figure 1.1. *A phylogenetic tree of microbial genomes. Branches represent different genomes, colors – different lineages, and bridges – events of HGT (Popa & Dagan 2011).*

Currently, several mechanisms of horizontal gene transfer are recognized (Fig. 1.2). The first one, called transformation, comprises all the events where free DNA is taken from the environment. Natural transformation is a form of transformation intrinsic to cells developing natural competence state without any extra-applied trigger. For such cells this process is a part of their growth cycle and is argued to be somehow customized for cells own needs. The evidence of the latter is that natural transformation is initi-

ated by the recipient cell rather than by donor as, for example, happens during conjugation. (Johnsborg et al. 2007; Popa & Dagan 2011)

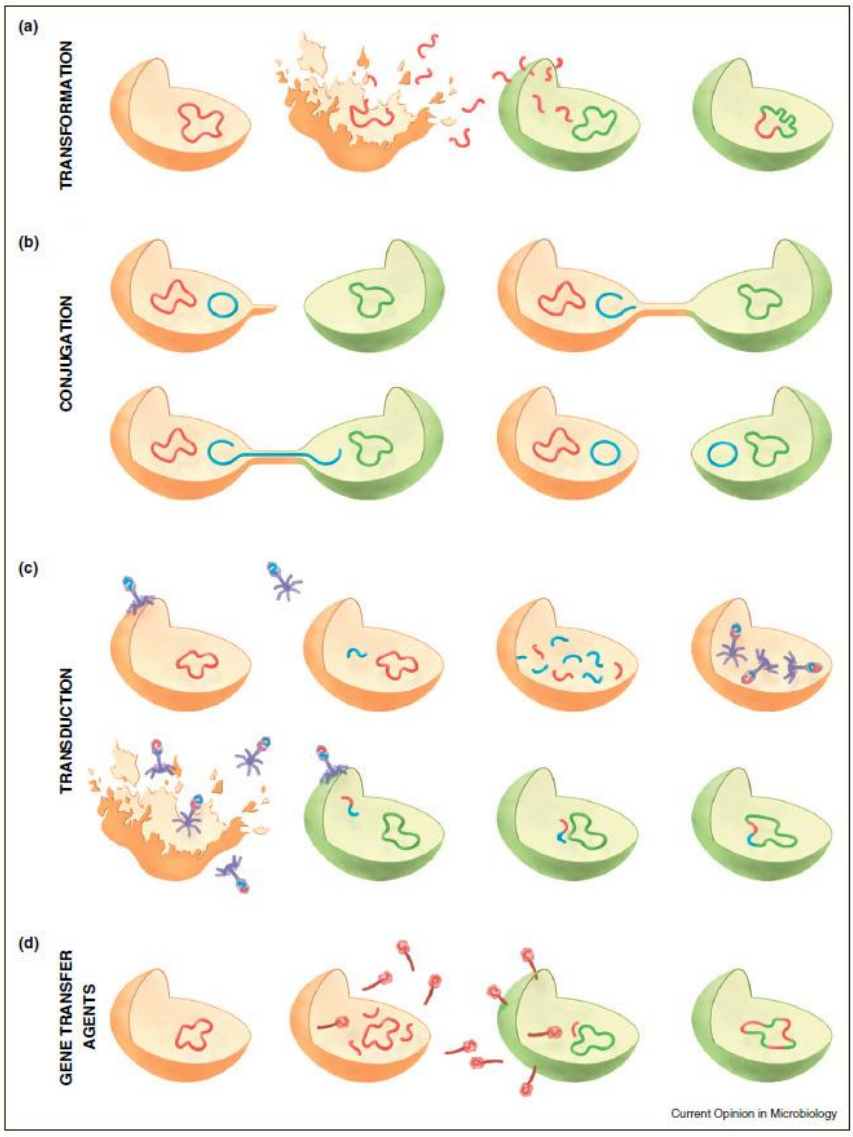

Figure 1.2. *Mechanisms of HGT. a) Naked DNA uptake from the environment; b) Plasmid transfer from donor to recipient cell by means of conjugation; c) Phages infect cells and can randomly take the pieces of host genetic information into own genome. During infection of the next host cell phage particles integrate the carried material including the one from previous hosts to the new host chromosome thereby conducting the gene transfer. The rate of gene transfer during transduction depends on the amount of cells able to survive the infection; d) gene transfer agents (GTAs) represent phage-like particles, which, in contrast to phages, do not initiate cell lysis and were observed to take part in transfer of genomic DNA only. Studies conducted with GTAs propose them as much more efficient mechanism of HGT than transformation or transduction (Popa & Dagan 2011).*

Conjugation is the other important mechanism of gene flow, where genes are spread on plasmids through specially formed protein tunnel tube formed between donor and recipient cells. Transition function is coded either by a part of the plasmid or by donor

cell genome. The third mechanism of gene transfer is mediated by phages or gene transfer agents and is referred to as transduction. (Popa & Dagan 2011)

Apart from the listed mechanisms of gene flow it is also interesting to focus on possible genetic material transfer directly through cell-to-cell contacts. Experiments on genetic material transfer between different species have revealed that for some cases cell-mediated gene transmission more likely resembles transformation rather than conjugation (Lorenz & Wackernagel 1994). It has previously been reported that naturally competent *Vibrio* sp. is able to uptake or receive nonconjugative broad-host-range plasmids from *E. coli* in the absence of helper plasmids (Paul et al. 1992). The authors obtained positive clones in liquid and filter transformations as well as no transformants in controls with DNase I, proving that mechanism of transfer is not conjugation. They have called this phenomenon natural plasmid transformation and proved the dependence of the transfer on cell-to-cell contact. Such, they demonstrated the absence of transformation in experiments where donor and recipient cells were separated by 0.2 μm filter as well as in experiments with *E. coli* supernatant as a source of transforming material. Both experiments were performed in presence of DNase I. (Paul et al. 1992) The other studies confirmed the occurrence of gene spread by such mechanism between the other species, as, for example, was recently demonstrated by Wang and colleagues (2007). The authors have observed the cell-contact mediated transfer of shuttle plasmid in DNase I sensitive manner from *E. coli* to *Bacillus subtilis*, that is, in a way distinct from conjugation mechanism. It appeared to be much more efficient on agar medium providing more chances for cell contact than in liquid cultures under the same conditions. (Wang et al. 2007)

Finally, one more mechanism contributing to all the mentioned previously was discovered recently by Dubey and Ben-Yehuda (2001), which can possibly be the explanation to gene transfer events observed in the aforementioned. According to these findings, special system of nanotubes (Fig. 1.3) might connect adjacent cells grown on solid surfaces creating a network for cellular communication through exchange of proteins, mRNA molecules, genetic information including nonconjugative plasmids and phages that, as was noticed earlier, may also provide new genes to the cells. (Dubey & Ben-Yehuda 2011)

It is additionally worth to mention the study by Neela and colleagues (2009), who have tried to investigate the possibility of tetracycline resistance transfer between marine and human enteric bacteria and came to interesting conclusions concerning its probable mechanism. This study proved the horizontal gene transfer between these bacteria during filter-mating experiments. It was shown that *tet*(M) gene can be transferred from chromosome of marine bacteria to *E. coli* and *Enterococcus faecalis* by cell-to-cell contact supposedly by newly formed plasmid-like element identified in several transformants. The authors initially suggested that the main role in *tet*-gene transfer would belong to Tn916 family transposon which was reported to assist tetracycline resistance transfer events (Agerso et al. 2002), however, in obtained transformants no transposon sequences were identified. (Neela et al. 2009)

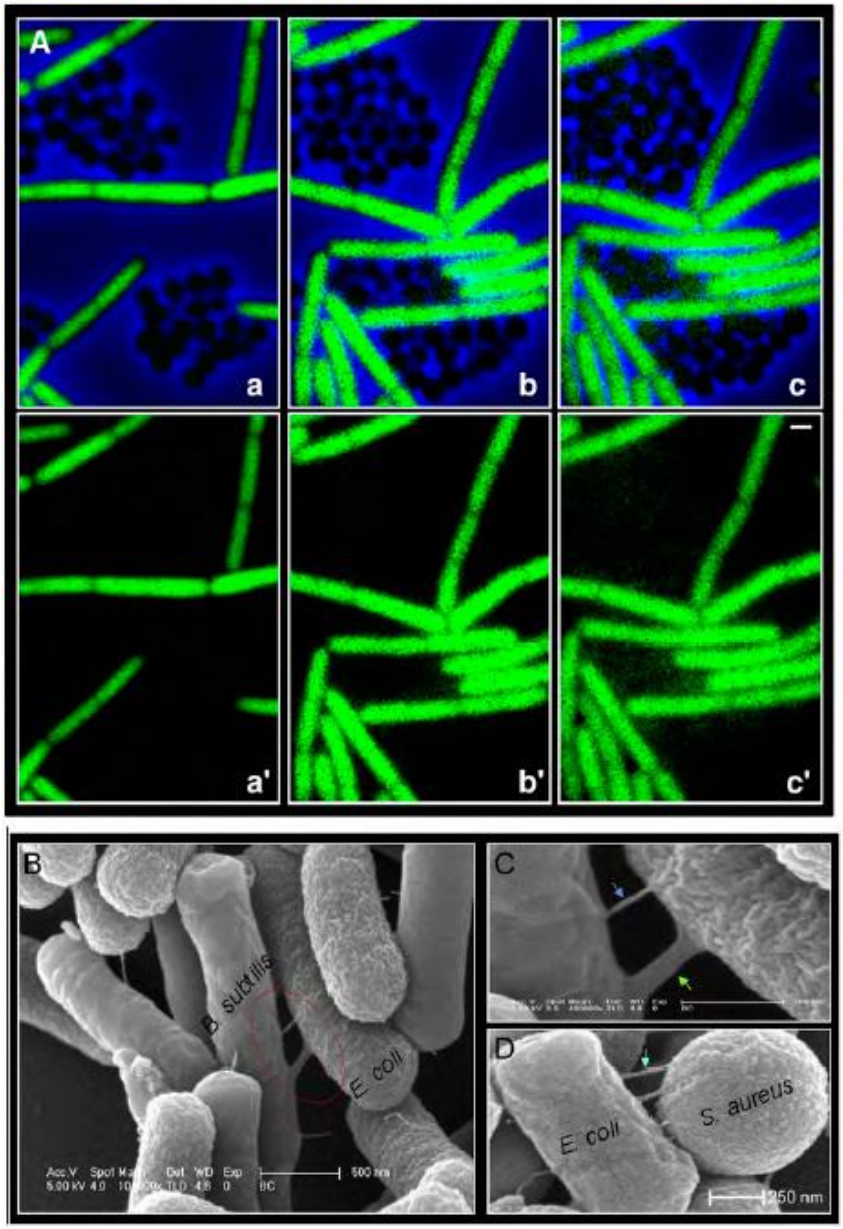

Figure 1.3. *Newly discovered mechanism of cellular communication including HGT through formation of membrane-like nanotubes between cells in community. (A) Pictures of Bacillus subtilis (chromosomal gfp+) and Staphylococcus aureus (gfp-) mixed in exponential phase and grown on the same agar plate. Pictures were made at 0 min (a), 30 min (b) and 50 min (c) after plating. From (a'- c') GFP and (a - c) overlay pictures it is clear that cytoplasmic GFP is gradually transferred from B. subtilis to adjacent S. aureus cells with the simultaneous decrease of fluorescence from gfp+ cells over time. (B,C,D) Establishment of this nanotubular network is proven to be not specific without reference to cell species. S. aureus, B. subtilis and E. coli cells visualized with-high resolution scanning electron microscope (HR-SEM) (x75,000). Interspecies tubes are indicated by arrows (Dubey & Ben-Yehuda 2011).*

Separation of cells by nitrocellulose membrane did not yield any transformants confirming necessity of cell contact. Authors propose that this might be a conjugative process; however, it remains uncertain whether pili structures are involved. Moreover, it was revealed that possibility of gene transfer is not only donor-mediated process, but for

the examined microorganisms depends on donor-recipient combination (Fig. 1.4). (Neela et al. 2009) All these findings indicate that horizontal gene transfer mechanisms are yet not fully investigated and there is a room for new unexpected mechanisms of gene transfer to be demonstrated.

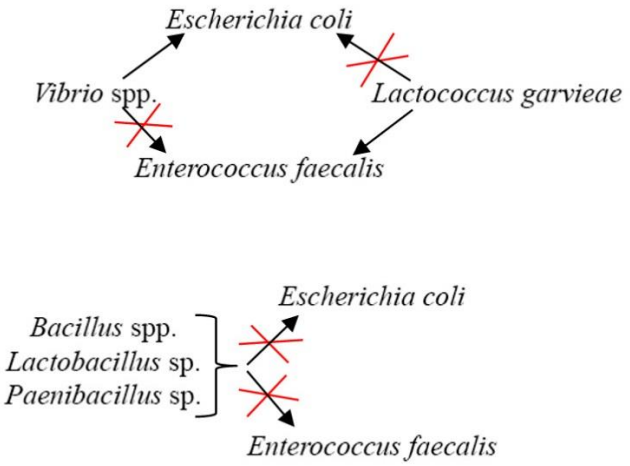

Figure 1.4. *Experiments revealing the horizontal transfer of tet gene from donor chromosome. Arrows represent the direction of gene transfer; red crosses indicate experiments where no transfer occurred.*

Many studies focus on HGT research in laboratory and till now some attempts were made to visualize it in environmental conditions. The rates of HGT are usually estimated and compared in laboratory using transformation frequency or transformation efficiency coefficients. The first is calculated as the number of bacteria with acquired DNA divided by the total bacteria exposed to that DNA in experiment. Transformation efficiency in its turn is calculated as the ratio of transformants number to amount of exposed DNA. However, these indexes don't reflect experimental conditions, dynamics of HGT event and, finally, can not predict the fate of acquired gene in population. Furthermore, laboratory methods have detection limits, which lead to overlooking of some fraction of HGT events that might still be important from an evolutionary perspective. (Thomas & Nielsen 2005)

In order to understand how important is the HGT in evolution it is necessary to estimate which amount of genetic information in organisms was acquired in that way. To date, it was evaluated that 1.6 to 32.6 % of the genome of the average microbe was acquired through HGT (Koonin et al. 2001) and about 66 to 96 % if consider its accumulative effect on lineages (Dagan et al. 2008). It was also concluded that over the evolution time all prokaryotes were affected by HGT, however, at a slow rate (Boto 2010; Dagan et al. 2008).

In general, there are many barriers towards HGT in nature. Several trends were discovered during the attempts to characterize the process and likelihood of HGT between different bacteria. First of all, it was shown that HGT occurs predominantly between closely related species with similar (>95%) GC content. Secondly, for gene transfer to

be possible, for example, by conjugation, it is important that the recipient and donor are at a close proximity to each other (Popa & Dagan 2011).

In case of natural transformation, where donor is raw DNA, stability of this molecule in the environment is crucial for the overall success of HGT (Fig 1.5). The higher is the DNA stability the higher is the time during which bacteria can handle the molecule and, hence, the higher is the transformation frequency. As it was shown previously, DNA can be stored in the environment for quite a long term and the approximate amount of free DNA in soil can reach 1 μg/g, while in marine water it ranges between 0.03 and 88 μg/l (Ogram et al. 1987; Deflaun & Paul 1989; Karl & Bailiff 1989, cited in Thomas & Nielsen 2005).

Additionally, latest phylogenomic studies confirmed that in the vast majority of cases (74%) the HGT occurs between organisms belonging to the same habitat (Hooper et al. 2009). Finally, the success of gene import and incorporation into genome do not guarantee its functionality in the recipient organism. For the acquired gene to be functional and transmitted to the next generations, it has to be incorporated in the genome either with its own recognizable by recipient promoter or to be inserted near the existing one. Plasmids also should have the ability to replicate in new host. Moreover, gene is expected to endow the recipient new valuable functions and increase its fitness in order to be retained (Fig. 1.5). (Thomas & Nielsen 2005; Popa & Dagan 2011)

Development of the natural competence state and it's maintenance is a process costly to the cell. It spends cellular and energy resources on synthesis of competence proteins and deals with gene incorporation consequences, which are not always beneficial. Therefore, some argue about the particular role of natural transformation for the cell.

There are two major points of view on natural transformation primary purpose. First observes this process as the mechanism of damaged regions repair, acquisition of novel functions and overall genetic diversification. Second theory suggests natural transformation as a mechanism for uptake of DNA as a nutrient. These two theories can, however, complement each other. (Johnsborg et al. 2007)

In addition to that, experiments show that different species develop the competence state in response to different factors, meaning that the primary purpose of natural transformation may vary between species (Johnsborg et al. 2007). Such, it was revealed that many species as, for example, *Streptococcus pneumoniae* and *Acinetobacter calcoaceticus* uptake only one strand of double stranded DNA (dsDNA), which would be considered as wasting behavior in terms of food capture. Moreover, definite cellular proteins including late competence proteins serve to protect the taken ssDNA from degradation and prepare it for recombination, which undoubtedly connects natural transformation primarily with acquisition of new genetic information. (Berge et al. 2003) The other fact confirming that natural transformation is not simply the mechanism of food gathering is the induction of competence in *A. calcoaceticus* after addition of fresh medium to old cultures, proving that cells have enough nutrients during developing of natural competence (Palmen et al. 1993).

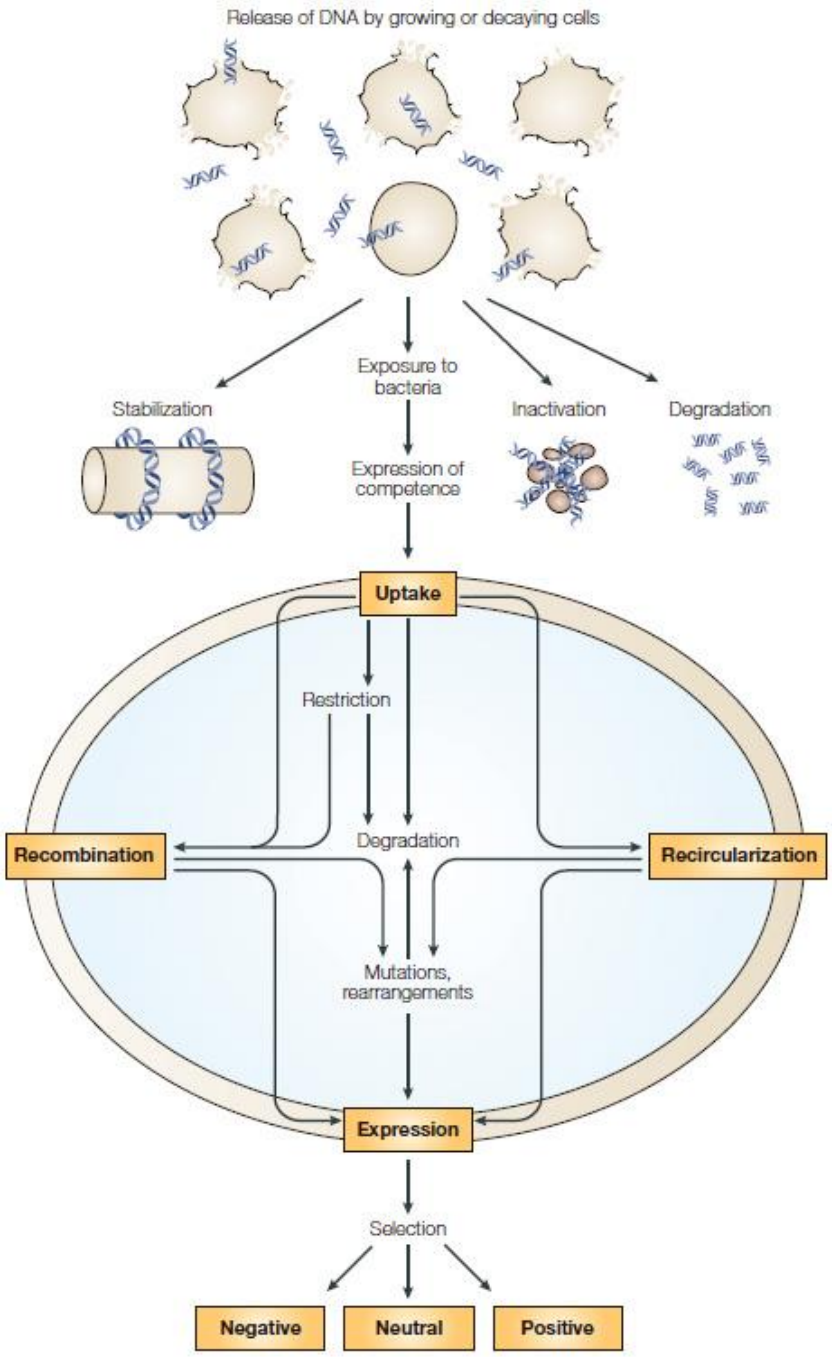

Figure 1.5. *Natural transformation steps. Free DNA originated from dead cells (passive release), naturally excreted in the environment by living cells (active release) or released in result of fratricide mechanism utilized by, for example, Streptococcus sp. (Johnsborg et al. 2007) is taken by a competent cell into its cytoplasm. There the molecule has to integrate into host chromosome or, in case of plasmid, recircularize, otherwise it undergoes degradation by host cell nucleases. Recombination or recircularization is followed by expression and selection on a basis of newly acquired function. If gene impact appeared to be negative or neutral, cells tend to get rid of such ballast introducing genetic changes or simply excluding the gene or its part (Thomas & Nielsen 2005).*

Many bacteria possess natural transformation mechanism and are widely used in laboratory. *A. baylyi* ADP1 is one of the most recognized among them.

3 *A. baylyi* ADP1 in natural transformation studies

Acinetobacter genus belongs to gamma-proteobacteria and is found in water and soil as well as occurs in some living organisms. *Acinetobacter* comprises Gram-negative bacteria occurring usually as immobile paired short bacilli often taking diplococcal shape during the stationary phase. Genus can grow utilizing diverse compounds as carbon and energy sources and can survive only in strict aerobic conditions. (Young et al. 2005)

Among all the *Acinetobacter* strains *A. baylyi* ADP1, which is used in the current study, is one of the most widely studied. Its genome has been fully sequenced and annotated almost one decade ago providing researchers with valuable knowledge on similarities with other model microorganisms and possibilities for future deep strain investigation (Barbe et al. 2004). Accordingly, this microbe has become widely employed in laboratories nowadays also due to the ease of handling, lack of pathogenicity and unique traits which make this organism the suitable model for metabolic studies and product engineering, bioremediation, investigations of lateral gene flow and other genetic experiments (Gerischer 2008; Metzgar et al. 2004).

A. baylyi ADP1 strain taxonomic position was officially confirmed by Vaneechoutte et al. (2006). ADP1 (previously named as BD413) was derived from soil inhabitant *Acinetobacter* sp. strain BD4 and is more widely used due to the smaller capsule that makes its laboratory handling much more convenient in comparison to parent strain. In addition to that, during the previous years the collective name of *A. calcoaceticus* was used in many studies and referred to both BD4 and ADP1 (Vaneechoutte et al. 2006). For the convenience of correlation with sources, in this review I will use the original names utilized in articles to refer to *A. baylyi* ADP1.

Previous studies have shown that three strains of *A. baylyi* species, namely ADP1, B2 and 93A2, possess the higher propensity to natural transformation demonstrating 100- to 1000-fold higher transformation frequencies than any other *Acinetobacter* strain (Palmen & Hellingwerf 1997; Young & Ornston 2001; Metzgar et al. 2004; Vaneechoutte et al. 2006). However, *A. baylyi* ADP1 was historically the first strain from the listed *Acinetobacter* strains known for its high competence and, therefore, was widely used as a model in studies concerning natural transformation throughout the last decade. The routine protocol for *A. baylyi* ADP1 natural transformation in laboratory was described in detail by Metzgar et al. (2004) with the emphasis on obvious advantages of this microorganism over the traditional Gram-negative model organism, *Escherichia coli*. Those include natural competence of *A. baylyi* ADP1 and intrinsic ability to recombine DNA regions to own chromosome based on homology, while *E.*

coli requires additional manipulations for DNA transposition through the cell membrane and supply of recombination function as well as suppression of cellular nucleases representing additional obstacle towards successful recombination (Metzgar et al. 2004). Additionally, it was noticed that *A. baylyi* ADP1 cells enter competent state almost immediately after supplement of fresh medium to the stationary phase culture. Transformation frequency peak was observed then within first three to five hours with subsequent gradual loose of competence as the carbon source was depleted. The interesting fact is that *Acinetobacter* sp. do not differentiate between the DNA of different origins and recombine both homologous and heterologous genes with the same frequencies, which broaden potential tools for natural transformation investigation. (Palmen et al. 1993)

According to the general mechanism of natural transformation, only one DNA strand is translocated through the membrane while the other is degraded. To ensure the successful acquisition of new genes bacterial cells protect the incoming DNA from degradation with the aid of specific binding proteins. For *A. baylyi* ADP1 such proteins have been suggested during the full genome annotation, however, not all of them yet been experimentally proven. (Gerischer 2008)

3.1 Structure of *A. baylyi* ADP1 DNA uptake machinery

Upon discovery of new proteins involved in *A. baylyi* transformation the additional experiments were performed to reveal the functions of particular proteins (Porstendorfer et al. 1997; Link et al. 1998; Busch et al. 1999; Herzberg et al. 2000; Porstendorfer et al. 2000; Friedrich et al. 2001). These include studies of mutant clones, biochemical and immunological analyses as well as comparison of found proteins against similar ones with proven function. As a summary of these experiments, it was noticed that *A. baylyi* competence proteins resemble type II protein secretion and type IV pili (Tfp) system proteins. Table 2.1 demonstrates sixteen competence proteins totally discovered for *A. baylyi* ADP1, their analogs in other well-studied microorganisms and deduced functions. (Gerischer 2008)

Until present, there were no studies, which would experimentally prove the whole structure of *Acinetobacter baylyi* ADP1 DNA uptake machinery. However, such a recognized scientist in the field of *Acinetobacter* as Beate Averhoff and her colleague Iris Graf have proposed such a scheme (Fig. 2.1) (2008). It was assumed that secretin-like ComQ proteins form homopolymeric ring structure with the tunnel for DNA pass in the outer *A. baylyi* ADP1 membrane. The hole can be static or alternatively be open only in response to special signals produced, for example, when the receptor protein ComC bounds DNA to the outer membrane. According to Averhoff, there are two possible further mechanisms of DNA translocation. One of them suggests that pilin-like ComP, ComE, ComB and ComF proteins are assembled into pseudopili and extend from the cytoplasmic to the outer membrane and back to guide the trapped DNA through the periplasmic space. The pseudopili movement can theoretically be achieved through the

process of polymerization/depolymerization with involved traffic ATPase (PilT) similar to NTPases characterized in other microorganisms such as *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. (Averhoff & Graf 2008)

The second possible mechanism of DNA transfer can involve ComEA protein, which can potentially carry the bound DNA from ComQ channel through the periplasm to the ComA channel in cytoplasmic membrane. In that case, pilin-like proteins can play the role of guiding path for ComEA protein and facilitate DNA translocation. ComA channel is comprised of ComA competence proteins reported to be shared by many Gram-positive and negative bacteria. (Chen & Dubnau 2004; Averhoff & Graf 2008)

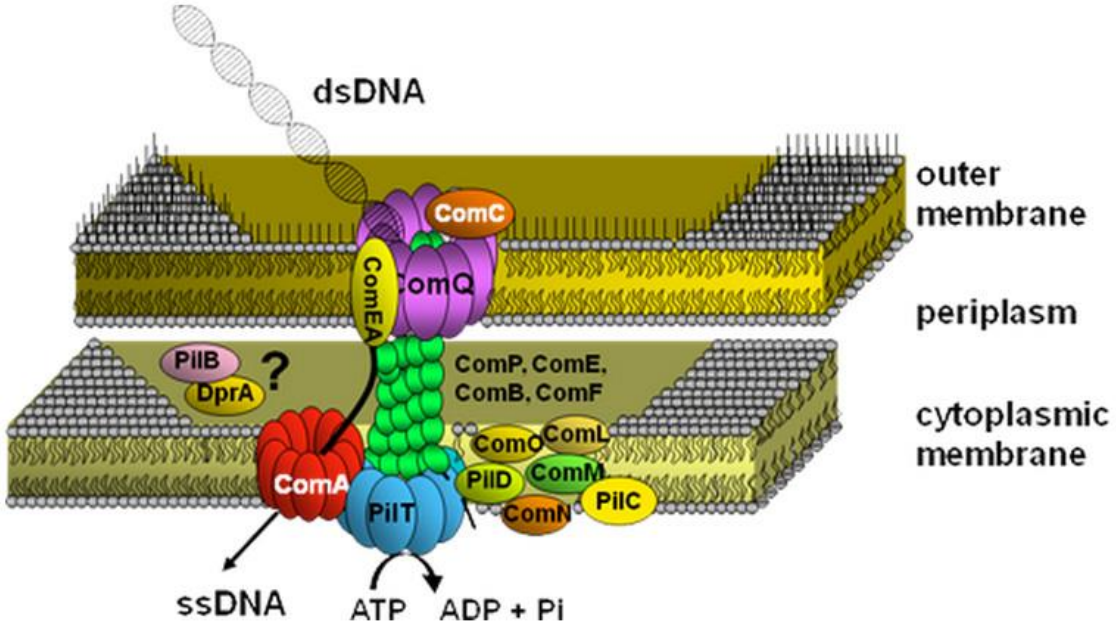

Figure 2.1. *Deduced structure of A. baylyi ADP1 uptake system. See text for detailed description (Averhoff & Graf 2008).*

The rest of the proteins depicted on Figure 2.1 ComO, ComL, ComM and ComN are suggested to facilitate the assembly of uptake machinery parts associated with the cytoplasmic membrane. Performed studies have shown that some of the members of discussed machinery are absolutely essential for natural transformation, for example, ComQ, ComA, ComP, ComB, ComEA and ComC, while others have not so drastic value for the process and can be missed with only partial loss of transformation potential, for instance, ComE and ComF. (Porstendorfer et al. 1997; Averhoff & Graf 2008)

Porstendörfer and colleagues performed the closer study of ComP protein and its synthesis in the cell (Porstendorfer et al. 2000). The obtained data showed that the peak of ComP expression occurred in the late stationary phase, which made researchers to suggest that DNA uptake machinery "bricks" are most probably already in the cell before induction of competence observed in many experiments immediately after addition of fresh medium. The activation of already existing system can explain the phenomenon of such a fast response. Taking into consideration energy-dependent nature of natural transformation, it was also suggested that DNA uptake function could be slowed down

with the maturation of culture and depletion of carbon source since there is less energy available for that process in the stationary growth phase. (Palmen et al. 1993; Porstendorfer et al. 2000)

Table 2.1. *Competence proteins of A. baylyi ADP1 (reviewed in Gerischer 2008).*

Acinetobacter competence proteins	Related proteins	Potential function
Group I (DNA translocator specific proteins)		
ComA	ComA (*N.g.*)	Transport of DNA through the inner membrane
ComEA	ComEA (*N.g.*)	DNA binding protein
DprA	DprA (*T.t.*)	DNA processing protein
Group II (type IV pili-related proteins)		
ComP, ComE, ComF, ComB	Prepilins (*N.g.*, *P.a.*)	Structural subunits of the DNA translocator, transport of DNA through the periplasm
ComC	PilC (*N.g.*)	DNA binding and/or transport
PilD	Prepilin-processing protease	Export and maturation of prepilins
PilC	PilC (*P.a.*)	Platform of the DNA translocator
PilB	PilB (*P.a.*)	Biogenesis of the DNA translocator
ComQ	Secretin	Translocation of DNA through the outer membrane
ComL	PilP (*P.a.*)	Biogenesis or stabilization of ComQ multimers
ComM	PilM (*N.g.*)	Biogenesis of the DNA translocator
ComN	PilN (*P.a.*)	Biogenesis of the DNA translocator
ComN	PilN (*P.a.*)	Biogenesis of the DNA translocator
N.g., *Neisseria gonorrhoeae*; *P.a.*, *Pseudomonas aeruginosa*; *T.t.*, *Thermus thermophilus*.		

Already ten years ago, researchers had some doubts about the role of pili in DNA uptake process in *Acinetobecter* sp. It was revealed that mutants carrying defective *comB*, *comE*, *comF* and *comP* genes had no noticeable defect in pili structure and function. Therefore, researchers inclined that pili must have not to be involved in natural transformation of *A. baylyi* ADP1 (Porstendorfer et al. 2000). This was subsequently confirmed by other research by Gohl and colleagues (2006). In this study authors have identified several genes and their corresponding products, including AcuA, as the major components of thin pili exhibited by *A. baylyi*, in absence of which no thin pili were formed. Mutants lacking thin pili were then examined on the capability to be naturally transformed. As a result, it was revealed that the transformation frequencies were identical to those of wild type strain. Accordingly, despite the fact that many proteins essen-

tial for natural transformation of *A. baylyi* ADP1 are pili-related proteins (Table 2.1), it has been concluded that type IV pili itself are not involved in this process. (Gohl et al. 2006)

3.2 Factors controlling natural transformation and mechanisms of integration of translocated genetic material into *A. baylyi* chromosome

As was already mentioned in the previous sections, natural transformation is the complex process. Many experiments were conducted to date to examine the impact of different factors on transformation. Among them is the research by De Vries and Wackernagel (2002), who performed the study in which they examined the influence of ***homological sequences*** on the ability of *Acinetobacter* sp. to recombine foreign DNA fragments. For the ease of analysis of transformants authors utilized the linear 3.72 kb donor DNA fragment carrying the intact KAN resistance gene and specially designed recipient plasmids rather than examine the recombination to the chromosome. Recipient plasmids were constructed to have either one (1096 bp) or two (915 bp and 945 bp) homology sites to the donor plasmid or not to have any at all; selection was performed based on restoration or acquisition of resistance to kanamycin. As a result, the study has revealed that presence of one homology region is sufficient to provide the anchor for illegitimate recombination (IR) of foreign DNA and have quite high frequency of transformation (Table 2.2). The frequency observed during experiments with one region of homology was 10^4-fold less than frequency of integration of the constructs with two homology sites. However, such constructs were recombined at least 10^5-fold more often than those having no homology regions. Totally IR events were finally not detected with the tested dilutions and transformation frequency was assumed to be less than $1.3 * 10^{-13}$ positive clones per DNA molecule. Additionally, both one- and two-region facilitated recombination events were proven to depend on RecA, protein playing the central role in homologous recombination, and lower transformation frequencies were exhibited by *recA* mutant clones. (de Vries & Wackernagel 2002)

Table 2.2. *The frequency of transformation of Acinetobacter with construct having two, one or none homology sites to the harbored plasmid. Frequencies are calculated as the amount of transformants per one molecule of added DNA (de Vries & Wackernagel 2002).*

Homology sites	Transformation frequency	
	Absolute	Relative
Two	$0.9 (\pm 0.1) * 10^{-4}$	1.0
One	$1.1 (\pm 0.03) * 10^{-8}$	$1.2 * 10^{-4}$
None	$\leq 1.3 * 10^{-13}$	$\leq 1.4 * 10^{-9}$

Further analysis of obtained transformants showed that single homology region of approximately 1kb in length can facilitate the IR of at least 2.6 kb of foreign DNA following the homologous site (Fig. 2.2). This event was called homology facilitated IR (HFIR). The mentioned length of incorporated DNA, however, was not limited by the natural capabilities of *Acinetobacter* but by the structure of recipient plasmid used in study, which had the origin of replication 2.9 kb off the homology region. In all transformants the amount of substituted bp did not match the amount of bp of incorporated foreign DNA. The regions of illegitimate recombination were random but had one same trend of possessing 3 to 8 nucleotide identity in the sites of fusion of donor and recipient DNA named microhomology regions. (de Vries & Wackernagel 2002)

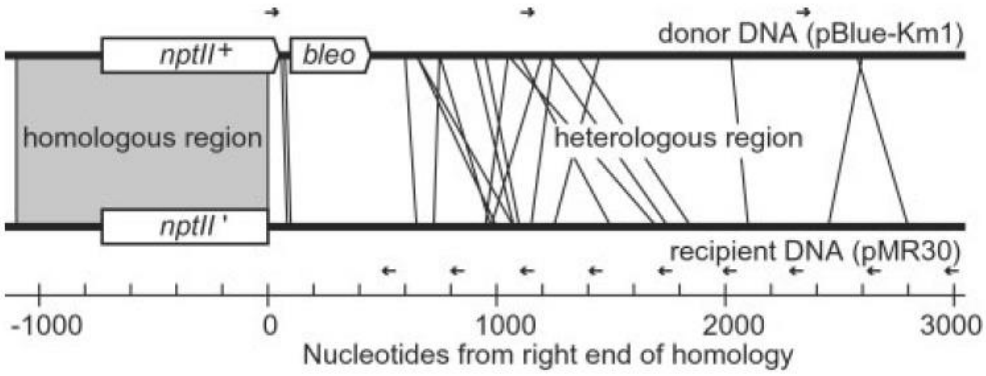

Figure 2.2. *The scheme depicting lengths and positions of foreign DNA incorporated by one homology region facilitated recombination in 20 random transformants. The lines represent the positions of borders between donor and recipient DNA for each clone. Arrows correspond to primers used for analysis of obtained recombined sequences. NptII – KAN resistance marker, bleo – bleomycin resistance marker (de Vries & Wackernagel 2002).*

In addition to presence of homology regions, authors have also tested the effect of the ***size of these homology*** regions on the transformation frequencies (Fig. 2.3). The obtained data indicated that 3.5-fold decrease in homologous sequence length entailed the 15-fold drop in transformation frequency, while 6-fold decrease (to 183 bp) – 300-fold drop. Nevertheless, the latter transformation was at least 500 times more efficient than transformation with the construct carrying no sequence homology. Further decrease of base pair number to 99 lead to transformation frequencies below the detection limit. (de Vries & Wackernagel 2002)

Further studies by Hülter and Wackernagel (2008) experimentally demonstrated both possible variations of illegitimate recombination in *A. baylyi*. IR in random location without any homology was named double illegitimate recombination (DIR). Homology-facilitated double illegitimate recombination (HFDIR), in contrast to HFIR, which the previous study was focused on, is a recombination event in which two heterologous sequences located from both sites of homologous region on the donor DNA are recombined into the recipient. (Hulter & Wackernagel 2008)

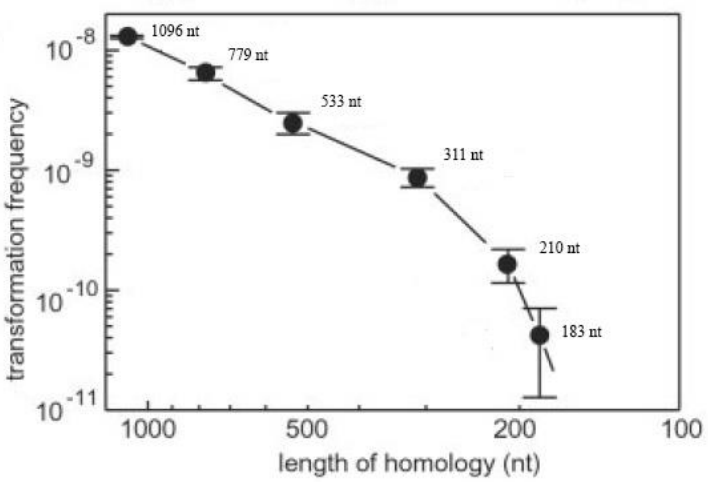

Figure 2.3. *The effect of the length of homology site on the transformation frequency of Acinetobacter baylyi BD4 (de Vries & Wackernagel 2002).*

The frequency of HFIR appeared to be four orders of magnitude higher than that of HFDIR, which is, obviously, the consequence of higher probability of single IR event than double. In addition to that, the analysis of microhomology regions in obtained transformants showed GC content of 74-79% which was suggested to facilitate HFDIR (Hulter & Wackernagel 2008), while only 53-63% content was observed to be sufficient for HFIR (de Vries et al. 2004).

It was also revealed that several of chosen transformants obtained both by HFDIR and HFIR used the same microhomology and extended microhomology regions but formed the fusions randomly in different parts of this regions (Fig. 2.4C). This have led the authors to conclusion that the first event taking place during IR can also be just hybridization between donor and recipient strands, then strand cut and subsequent formation of covalent bond. (Hulter & Wackernagel 2008) This is opposed to the previous suggestions about the recombination in the homologous region to be the first step to the IR events, which in that case would be facilitated by close proximity of two DNA strands (de Vries & Wackernagel 2002).

Furthermore, researchers have obtained the first reported *A. baylyi* transformant resulted from fully IR and the frequency of such event was $7.3*10^{-13}$ per cell. Analysis of resulted fusion sites demonstrated that 2258 bp fragment containing KAN resistance marker integrated into chromosome by accident, in absence of any microhomology regions (Fig. 2.4AB). DNA gyrase and topoisomerase I can catalyze such homology-free events by mechanism, which can involve double-strand breaks (Kickstein et al.2007) and yet remains to be fully clarified. (Hulter & Wackernagel 2008)

Interestingly, the other team of scientists has performed the assays aimed to find interconnection not only between transformation frequency and ***length of flanking regions*** but also to cover the variation in the ***insert size*** as a factor influencing transformation frequency (Simpson et al. 2007). It was revealed that the size of the insert does not play any role if the flanking homology is genome long, i.e. when cell lysates are used for transformation. For explanation of experiments with shorter homology regions

authors have found more useful to utilize total homology length indicator along with the length of each flank. For constructs containing less than 500 bp of total or flank homology and intended to incorporate 434 bp insert either in genome or in plasmid the transformation frequencies appeared to be quite low and spread randomly, without no visible correlation with homology length. However, when flanking regions were above 500 bp there was a trend of linear increase of frequency with increase of homology length up to 2000 bp. (Simpson et al. 2007)

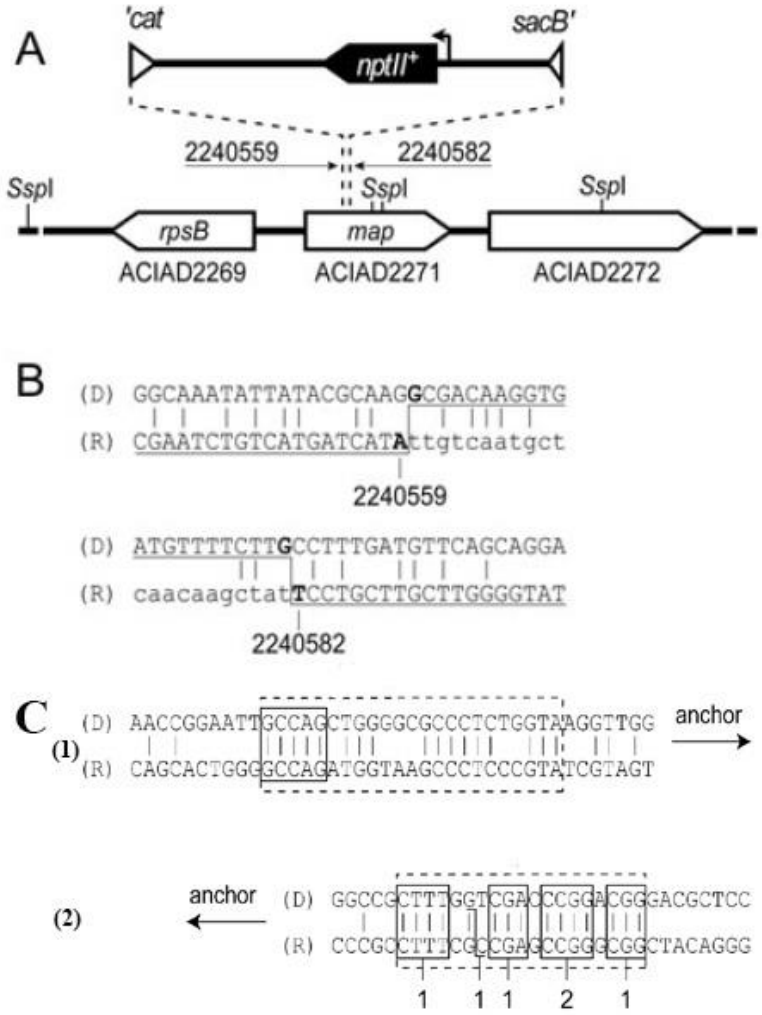

Figure 2.4. *(A) The location of 2258 bp sequence inserted in the map gene of A. baylyi by DIR with exclusion of 22 bp; (B) Incorporated fragment and genomic DNA fusion points depicting that IR has occurred between highly divergent sites; (C1) Example of "hot spot" microhomology (boxed by continuous line) proved to promote the fusion in several transformants out of all observed; (C2) Extended microhomology (dashed line), different parts of which were used by transformants during IR. Numbers indicate the amount of transformants with fusion at respective microhomology site. (D) – donor DNA sequence; (R) – recipient DNA sequence (Hulter & Wackernagel 2008).*

Researchers have also found that for the larger inserts (773 and 2400 bp) increase of total homology length in the range of 1974 to 11195 bp was accompanied with increase in transformation frequency. However, for 773 bp insert this increase was considered

statistically not significantly different from the genomic DNA control. This means that the correlation between homology length (larger than 500 bp for each flank) and transformation frequency takes place only for the large inserts. Additionally, transformation frequency at any particular flanks' size was obviously higher for the smaller insert, indicating that braced sequence size is also a variable affecting the final efficiency. (Simpson et al. 2007)

In result of this study, the authors have suggested the formula (1) for the mean transformation frequency (*Tf*) predictions based on ratio of total homology to the size of insert (R_{hi}), size of the smallest of the two flanks (H_{min}) and the total size of fragment supposed to be incorporated (F_{tot}). The equation is true for experiments with saturating DNA concentrations, and the curve, which the equation describes, is shown on Figure 2.5.

$$\mathrm{Log}_{10}Tf = -6.73 + 0.343\,R_{hi} + 0.00196\,H_{min} - 0.000567\,F_{tot} \qquad (1)$$

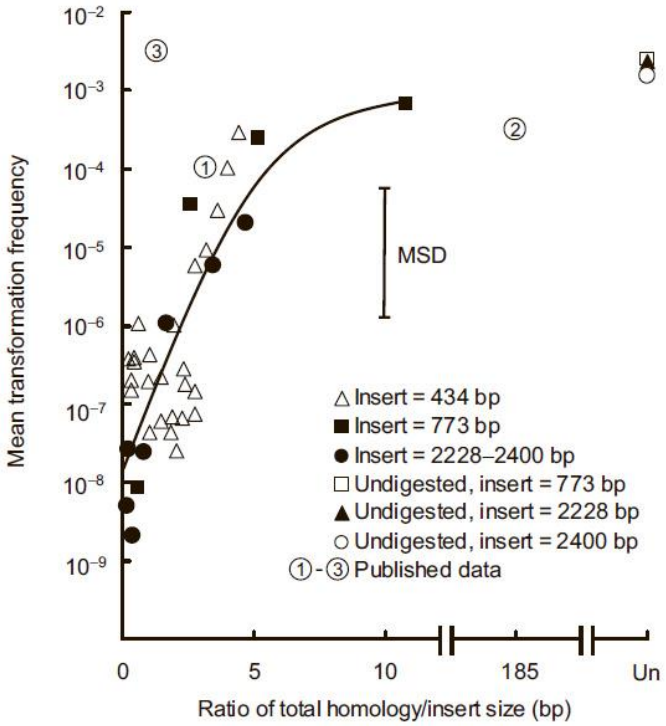

Figure 2.5. *Effect of the ratio of total homology to insert size on transformation frequency of A. baylyi BD413 (the plotted set of data includes the experiments with incorporation of constructs both in genome and in plasmid). Control experiments measurements for inserts flanked by genomic DNA were plotted separately due to inability to estimate the flanking size accurately (Un on X-axis). The published data added to the graph are: 1 – (Gebhard & Smalla 1998); 2 – (de Vries & Wackernagel 2002); 3 – (Stratz et al. 1996). MSD – minimum significant difference from the Tukey-Kramer test after analysis of variance (Simpson et al. 2007).*

3.3 Additional factors affecting the natural transformation

Palmen and colleagues in early 90th performed the series of experiments characterizing the natural transformation process in *A. calcoaceticus*. Their research has shown that the ***DNA form*** significantly affects the transformation frequency. So introduction of plasmid linearized by single enzyme digestion to the competent *A. calcoaceticus* cells resulted in 1000- to 10000-fold decrease in transformation events in comparison with intact plasmid or chromosomal DNA. Simultaneously, the mixture of plasmids linearized by two different enzymes had slightly higher transformation potential, which led to conclusion that by some means the presence of linearized plasmid variations facilitates the recircularization of the initial plasmid. (Palmen et al. 1993)

Apart from the form of introduced ***genetic material***, ***its size*** was also very important. It was shown that the transformation frequencies of *A. calcoaceticus* increased with increase of DNA fragment size in the range from 300 bp to 3800 bp (Fig. 2.6). Figure 2.6 also demonstrates that 1000 bp is a "border" size of DNA fragments, reduction below which results in fast drop of efficiency of transformation. Additionally, it was revealed that exposure to DNA with the size less than 600 bp lead to very rare events of successful transformation presumably because of exonuclease activity which generally destroys approximately 500 bp of each DNA molecule. Authors have also concluded that statistically only approximately 0.13% of all DNA internalized by cell was finally recombined into genome, while the rest was degraded. Previous studies demonstrated that in contrast to other transformable bacteria DNA internalization by *A. calcoaceticus* is linear during 2-3 hours, indicating that new DNA uptake spots might appear during this time. (Palmen & Hellingwerf 1997).

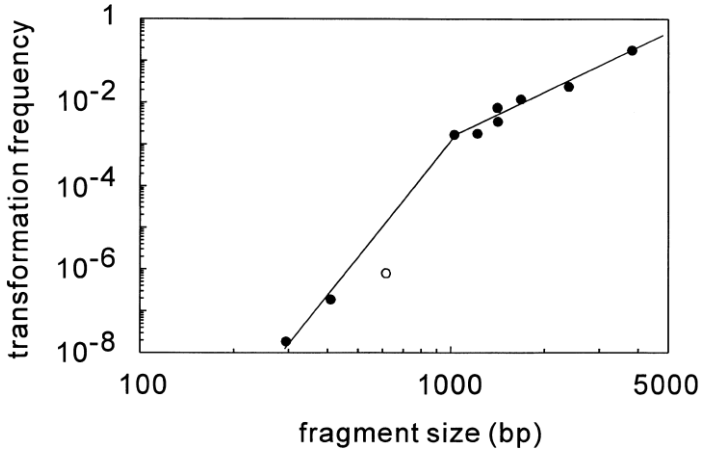

Figure 2.6. *The effect of DNA size on transformation frequency of A. calcoaceticus (Palmen & Hellingwerf 1997).*

The ***amount of introduced DNA*** undoubtedly has also an effect on transformation frequency. The effect of DNA concentration on frequency of *A. calcoaceticus* transformation corresponds to saturation model, in which further increase of DNA concentration over some point does not result in significant changing of frequency (Fig. 2.7). It

was also revealed that there is no connection between the DNA form and expected saturation concentration. Such, experiments showed that one of the examined plasmids saturated at 1-2 μg/ml of cells, while the other had not even at 20 μg/ml. (Palmen et al. 1993)

Furthermore, Palmen and colleagues examined the effect of ***pH*** on transformation and concluded that from the 5-8 pH range, in which *A. calcoaceticus* is able to grow, pH lower than 6.5 inhibits the transformation, but not the induction of competence. It was also shown that particular ***divalent cations*** had the effect on transformation as well. For example, treatment of mixture with EDTA stopped transformation almost completely. Return of Mg^{2+} and Ca^{2+} into solution increased the transformation level to 7%, while resuspension of cells in minimal medium without carbon source and in LB medium gave 7.7 and 31% restoration of transformation respectively. Additional experiments lead to conclusion that presence of carbon source did not affect the transformation level and could not explain the difference between cell behaviors in two examined mediums leaving this question open. It was, however, possible to assume that since Mg^{2+} and Ca^{2+} are necessary for transformation this may be associated with membrane-located nuclease activity. In addition to that, researchers observed the negative effect of Zn^{2+} particularly on transformation. (Palmen et al. 1993)

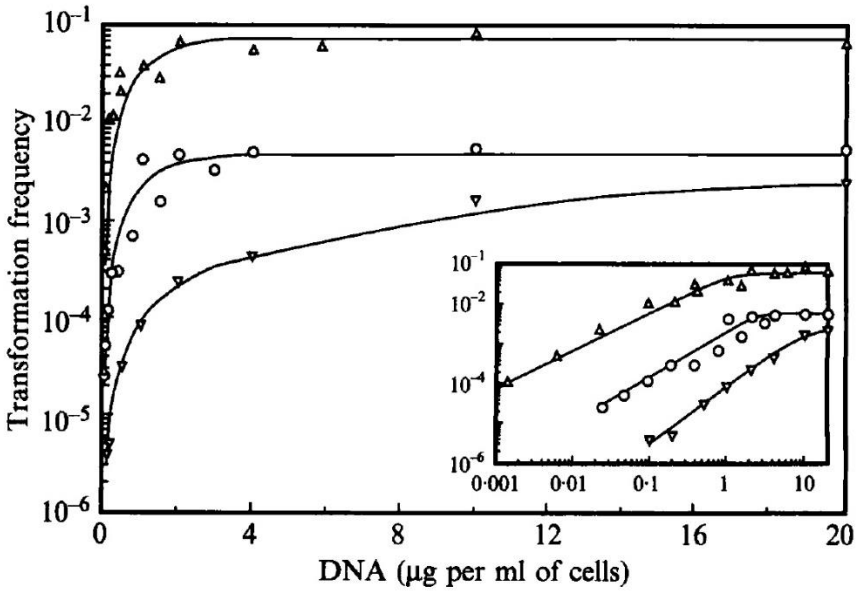

Figure 2.7. *Effect of DNA concentration on transformation frequency of A. calcoaceticus BD413. Circles represent chromosomal DNA; up triangle – plasmid containing cassette for homologous recombination; down triangle – plasmid replicating in Acinetobacter sp. On the insert DNA concentration is also plotted on a logarithmic scale (Palmen et al. 1993).*

Palmen et al. also tested transformation frequency against the ***time of incubation*** of competent cells with DNA. For this experiment, they used the vector carrying the cassette with kanamycin resistance gene confined within flanking regions for homologous recombination. Authors stated that they could identify transformants with plating meth-

od and kanamycin selection already after one minute. However, after addition of DNAse I and before plating the mixtures were additionally incubated for one hour to allow enough time for expression of resistance marker. (Palmen et al. 1993) This could lead to potentially wrong conclusions, since during the additional hour the transformation process, which could only start within the first minutes of experiment, can be further completed. Moreover, as the culture is in the exponential growth phase, the hour of cultivation is enough to increase the population several folds including the transformed individuals.

The same study proved that presence of ***genetic material competing for transformation*** reduced the frequency and efficiency of transformation by the target DNA. Since foreign and own genomic DNA acted as competing with the plasmid DNA, authors have concluded that, first of all, both types of DNA are processed by single type of uptake system. Secondly, due to the similar levels of inhibition for equal concentrations of genomic DNA from different sources it was assumed that *A. calcoaceticus* uptake machinery is not tuned to differentiate between them, which most probably is advantage rather than vice versa. (Palmen et al. 1993)

Additionally, Palmen and colleagues demonstrated that plasmids designed to recombine the target region into *Acinetobacter* chromosome through homologous recombination have higher potential for transformation than plasmids intended for maintenance (Fig. 2.7). Obtained results indicated that generally the process of recombination is more efficient than plasmid recircularization. (Palmen et al. 1993)

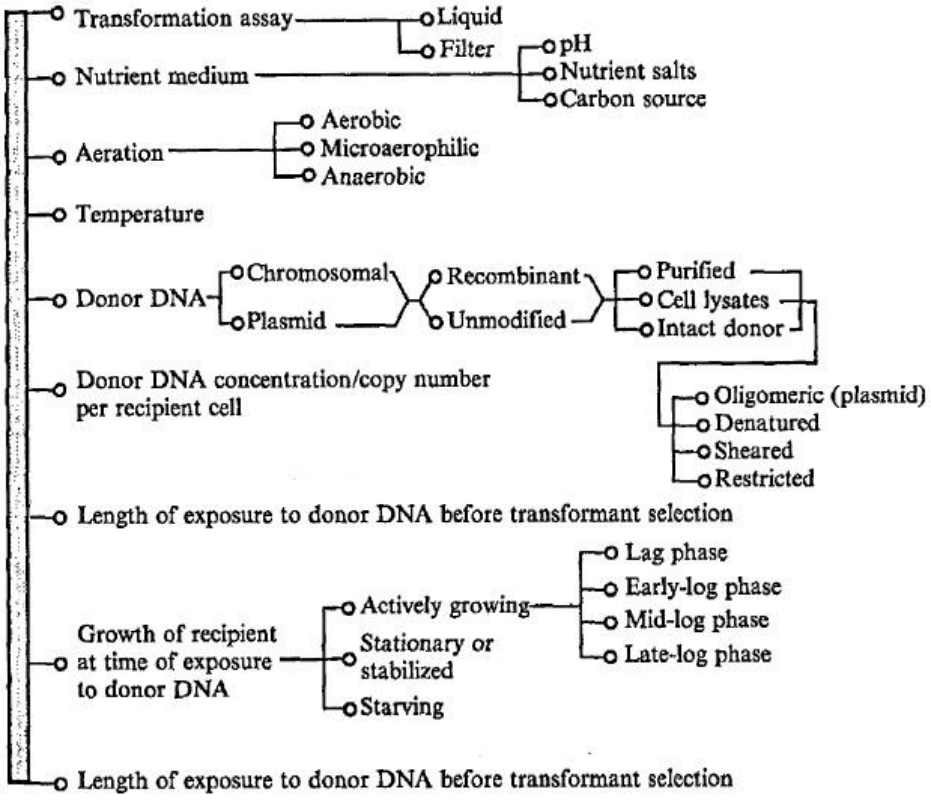

Figure 2.8. *Factors affecting transformation in in vitro experiments (Ray & Nielsen 2005).*

In general, parameters which deserve particular attention are summarized on Figure 2.8 by Ray and Nielsen, who gave the tips for planning natural transformation experiments. According to them, in addition to already mentioned issues, it is important also to take into consideration such factors as the growth phase, in which the recipient is during the exposure to DNA, growth conditions as well as nutrient source. (Ray & Nielsen 2005)

4 Methods for assaying natural transformation and horizontal gene transfer

The major scheme of all methods for assaying natural transformation comprises addition of DNA source to examined recipient and subsequent selection to distinguish the resulting transformants from the rest of population. The crucial feature for any selection method is the use of the selection mode suitable for purposes of particular experiment and allowing sensitive and effective identification of transformants. Detectable phenotypic changes can occur as a result of simple acquisition of marker genes or involving other more complex techniques as for example, the marker rescue technique, where desired genotype, and hence, phenotype is obtained via homologous recombination of intact donor gene with corresponding recipient gene damaged in advance. (Ray & Nielsen 2005)

4.1 Conventional method

Conventional methods for assaying natural transformation are culture-based, i.e. require cultivation in presence of selective pressure. The most widespread selection markers for investigation of gene transfer are antibiotic resistance markers, simply assigning the transformants with ability to grow on selective media. Utilization of markers endowing resistance to metal ions is also a variation of conferring advantageous phenotype for selection (Baulard et al. 1995, cited in Ray & Nielsen 2005). The other genes introducing to the transformants different metabolic traits can be utilized in transformation assays as the secondary markers, for example, protease activity (Wang et al. 2007). Plating method, however, possesses some limitations and disadvantages which will be discussed in more detail in the next sections. One of the main limitations is the inability of plating approach to reliably elicit the gene transfer dynamics and make real time following of the process more obvious. Therefore, during the last two decades the other biotechnology instruments have been adopted and applied for the purposes of HGT investigation allowing more freedom and possibilities than conventional method.

4.2 Attempts to visualize horizontal gene transfer in *A. baylyi* and other species

While many studies were devoted to revealing interconnections between various parameters and natural transformation frequency of *A. baylyi*, there are only few targeted on observing of transformation in dynamic in real time and visualization of this event.

Meanwhile, there is the growing concern on transgene dissemination from decaying transgenic plants by means of HGT to bacteria, and *Acinetobacter* sp. is one of the candidates for this role with the proven ability to contribute a lot to this process.

One of the attempts to employ the reporter genes for better visualization of HGT was made in 1996 by Jaenecke and colleagues. They utilized engineered *lac*Z gene coding for β-galactosidase activity as a reporter in experiments with conjugal gene transfer between different *P. putida* strains. In donor cells the reporter gene located on the high-copy plasmid was repressed both on translational and transcriptional levels from gene cassettes accommodated chromosomally so that colonies on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) substrate appeared white. Recipient cells grown on the same medium had the same white color, however, in the cases of conjugal plasmid transfer from donor to recipient, where the reporter gene happened to escape the repression, the colonies obtained the blue color due to β-galactosidase activity (Fig. 3.1). Such a system allowed the identification of gene transfer without use of selective medium and obtained transformation frequencies were shown to be of the same order of magnitude as on the medium with antibiotic. Finally, authors suggested the use of other reporter genes, such as luciferase and GFP, for further development of the strategy to track the flow of genetic material in environment. (Jaenecke et al. 1996)

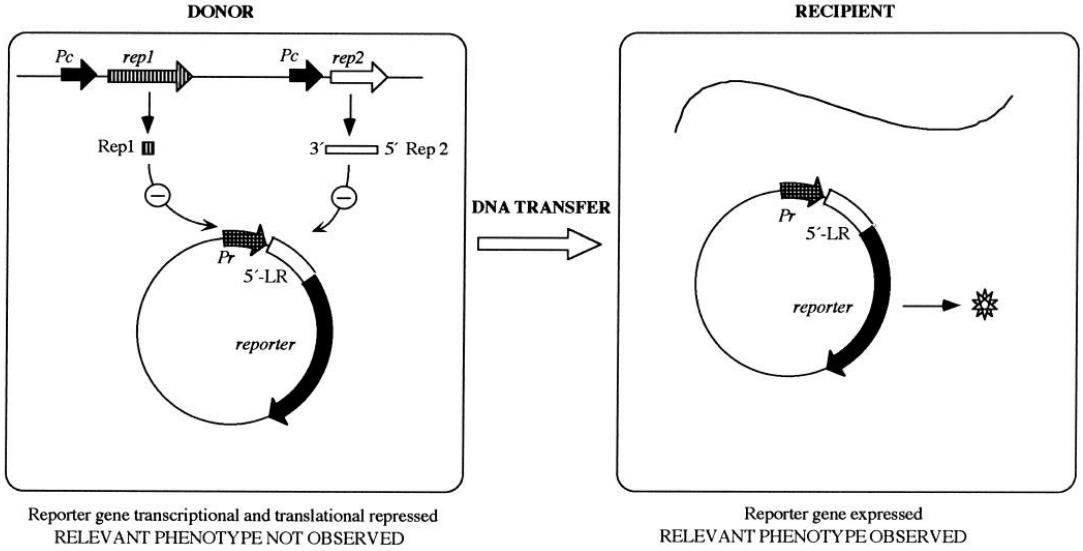

Figure 3.1. *The scheme of functioning of genetic circuit for studying horizontal gene transfer. The reporter gene is harbored on the highly mobilizable plasmid under broad host range tight promoter (Pr) and 5'-non-coding leader region (5'-LR). In the donor cells reporter gene is repressed on the level of transcription by rep1 and the level of translation by rep2 coding for antisense RNA to 5'-LR. Both repressors are constantly expressed from chromosome under constitutive broad host range promoters Pc. When plasmid is transferred by any mechanism to the cell lacking the repressor system reporter gene is expressed and conferring the cell definite phenotype (Jaenecke et al. 1996).*

This approach, however, is dependent on the quantity of repressor molecules and has a disadvantage of being prone to error in case of mutation in repression system as each separately taken repressor doesn't provide the full reporter gene silencing (Jaenecke et al. 1996). It is also limited to observation of transformation rates only after cultivation as is with conventional method, thereby making it possible to detect only culturable transformants, avoiding, however, the problem of loss of positive clones not readily culturable on medium with antibiotic.

4.2.1 Fluorescence reporter systems

Employment of fluorescent reporter genes as markers during transformation assays is accepted as very convenient approach since in contrast to conventional resistance-based markers it allows revealing the presence of transformants without additional culturing. Use of different fluorescent markers simultaneously also has a potential to give more information about donor and recipient interactions as well as transfer of different constructs in the same time frames during horizontal gene transfer. (Haagensen et al. 2002)

There are several studies focusing on HGT between plants and bacteria which visually demonstrate this process. Among them are the works by Rizzi, Pontiroli and colleagues (2008; 2009) who have clearly confirmed the ability of *A. baylyi* BD413 to uptake the plant DNA during growth on decaying plant tissues. The authors have developed the strategy for HGT direct visualization, which involves restoration of GFP expression in bacteria by means of acquisition of promoter region as result of the natural transformation with plant, bacterial chromosomal or plasmid DNA providing this repair sequence. The resulting transformants in that case can be directly visualized by fluorescent microscopy providing the horizontal gene transfer evidence. In addition to that, experiments with plant DNA have revealed the transformation hot spots across the different plant surfaces, which was previously difficult to accomplish. Moreover, the new strategy have avoided the selection plating stage which was the limiting step in the previous studies leading to sample's disturbance and, finally, depletion. (Rizzi et al. 2008; Pontiroli et al. 2009)

In experiments with transformation of *A. baylyi* BD413 with plasmid DNA from *E. coli* lysates, which were performed on filters, transformation frequencies were determined not only by conventional plating method but also based on cell fluorescence by means of confocal laser scanning microscopy (CLSM). Ten microscopic fields of equal size were randomly chosen for that purpose. Then the average surface area of bacterial cell was estimated by averaging the areas of 15 random fluorescent spots. This value was utilized for approximate quantification of transformants, while the total bacterial count in each area was calculated as the total area divided by estimated bacterial surface area assuming that cells grow in a monolayer. Finally, transformation frequencies were calculated as transformants per total count and averaged. Comparison of transformation frequency values obtained by plating and CLSM revealed that the latter is 10^2-fold higher (Rizzi et al. 2008), which was also the case for other culture-independent study

reporting the frequencies of HGT by conjugation (Sorensen et al. 2003; Sorensen et al. 2005). Authors suggest that one of the possible reasons for such a divergence in frequencies is inability of some recent GFP-positive clones to cope readily with concentrations of antibiotics used for plate selection. (Rizzi et al. 2008)

Musovic and colleagues (2006) have also performed culture-independent experiments revealing conjugative plasmid exchange between introduced donor *E. coli* cells and indigenous barley rhizosphere bacteria. For identification they used the approach utilized in a range of other similar studies (Dahlberg et al. 1998a; Haagensen et al. 2002; Sorensen et al. 2003) plasmid tagged with *gfp* sequence which was supposed to be expressed and detected only form transconjugants while being repressed in donor cells by chromosomally located repressor (Fig. 3.2). (Musovic et al. 2006)

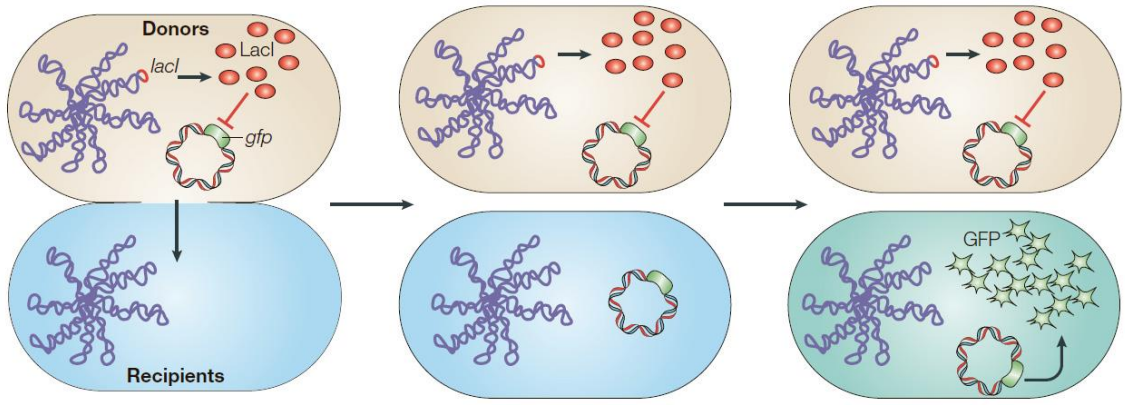

Figure 3.2. *The principal scheme of reporter gene transfer approach. The donor cells carry the fluorescent reporter gene harbored on the conjugative plasmid. The donor itself does not produce the reporter protein since its expression is blocked from the repressor gene localized on the donor chromosome. As soon as the plasmid is transferred to the donor cells lacking the repressor the active expression of the reporter gene begins leading to accumulation of fluorescent protein and easy detection of the resulting transconjugants (Sorensen et al. 2005). This or similar strategy for detection of conjugative plasmid transfer has been employed by a number of other studies (Christensen et al. 1996; Christensen et al. 1998; (Dahlberg et al. 1998b; Geisenberger et al. 1999).*

In parallel, authors have estimated the quantity of donor, recipient and total amount of cells by means of flow cytometry (Fig. 3.3). Moreover, cells were automatically sorted and used for identification of genera, which undergone transformation, through 16S rRNA sequencing of corresponding fraction lysate. Among the determined transconjugants the *A. calcoaceticus* was mentioned to be present in one of three independent HGT experiments. The authors do not suggest possible mechanism of plasmid transfer to *Acinetobacter*, emphasizing, however, that transferred plasmid belongs to plasmids generally transferred by conjugation. (Musovic et al. 2006) *Acinetobacter* sp. was also identified among transformants in other similar studies. For example, the research by Geisenberg and colleagues (1999) explored the transfer of conjugative plasmid in activated sludge environment utilizing the stability of GFP to fixation procedure. Transconjugants were further determined by whole cell hybridization (Geisenberger et al. 1999).

Flow cytometry employed in previous studies is an attractive method for convenient detection and enumeration of transformants expressing fluorescent proteins. It has apparent advantages over conventional cultivation-based methods: it is fast, much less laborious and much more precise because avoids the steps of dilution, cultivation on selective medium and "manual" enumeration of transformants, all stages of which affect the final result. It additionally allows fluorescence-activated cell sorting for further analysis of positive transformants, which is especially valuable for indigenous bacterial communities with unknown members. (Sorensen et al. 2003)

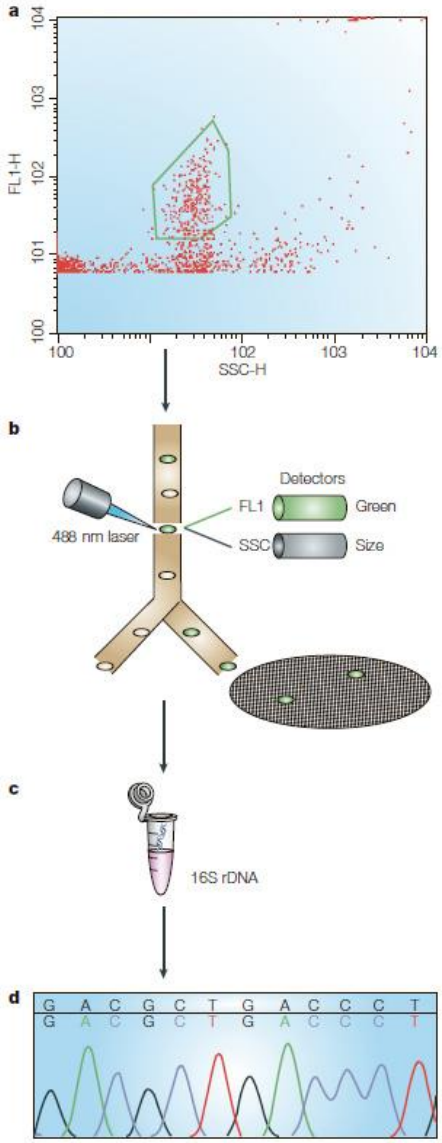

Figure 3.3. *Detection and sorting of fluorescence-positive cells resulted from horizontal gene transfer using flow cytometry and subsequent analysis of obtained transconjugants: (a) dot-plot of all the detected events of fluorescence in the coordinates of green fluorescence (FL1) versus side scatter (SSC); (b) cells meeting the constraints (green gate on the plot (a)) are isolated by fluorescent activated cell sorting onto membrane filters; (c) DNA isolated from the obtained cells is amplified and (d) sequenced providing the information about phylogeny of resulting transconjugants (Sorensen et al. 2005).*

4.2.2 Luminescence reporter systems

Luminescence genes are another marker genes widely used in biotechnology. Production of luminescence provides fast, sensitive and highly specific mean for identification of organisms with particular features. The main component of luminescent reaction is the luciferase enzyme, which catalyzes the conversion of specific substrate accompanied with light emission. Several types of luciferases catalyzing emission of light of different wavelengths have been discovered in the last time, however, the most wide used in experiments with bacteria are still bacterial (*lux*) and firefly (*luc*) luciferases. (Prosser et al. 2000; Badr & Tannous 2011)

The substrate of bacterial luciferase is the long chain aldehyde (naturally, tetradecanal), which is oxidized to the corresponding fatty acid in the process of the following reaction resulting in blue-green light production:

$$RCHO + O_2 + FMNH_2 \rightarrow RCOOH + FMN + H_2O + light \quad (2)$$

Reduced flavin mononucleotide ($FMNH_2$) is another compulsory component, oxidized to flavin mononucleotide (FMN), and is a potential limiting factor for the light emission. Additionally, bacterial luciferase is able to recognize the other long chain aldehydes, such as nonanal and decanal, and their conversion may also contribute to luminescence production. In the absence of any substrate, so-called "dark decay" takes place resulting in formation of FMN and H_2O_2 without any light emission (Fig. 3.4). (Meighen 1991; Prosser et al. 2000)

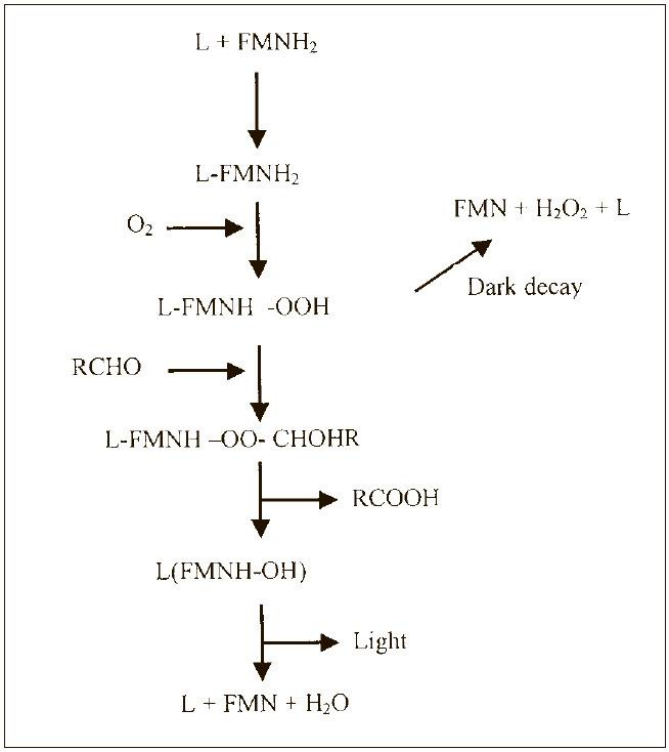

Figure 3.4. *Reaction of luminescence production depicted with major intermediates. L – luciferase enzyme (Prosser et al. 2000).*

Bacterial luciferase operon consists of five major genes *luxCDABE* (Fig. 3.5). *LuxA* and *luxB* genes code for α- and β-subunits of luciferase and were proven to be highly identical to each other. However, α-subunit is identified as the crucial for enzymatic activity, while β-subunit plays most likely stabilizing role in enzyme conformation. The rest of the operon encoding the fatty acid reductase complex is responsible for the substrate production from available fatty acid biosynthesis precursors, such as tetradecanoyl-acyl-carrier protein (ACP). Conversion of this precursor to aldehyde substrate is ATP- and NADPH-dependent, while emission of light is dependent on $FMNH_2$ presence. (Meighen 1991; Meighen 1993; Prosser et al. 2000)

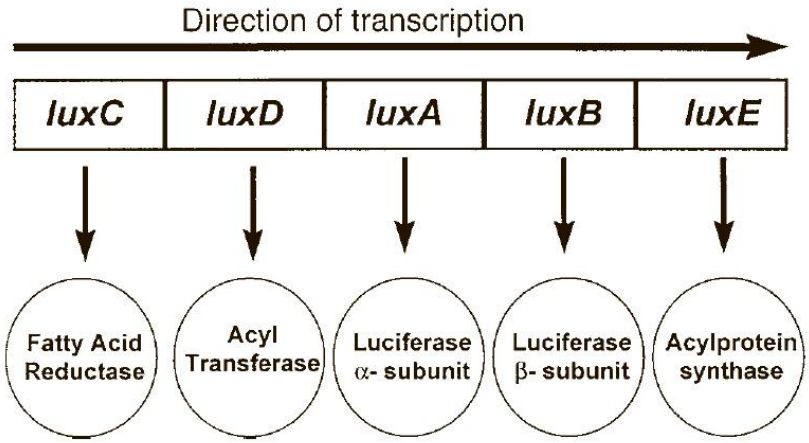

Figure 3.5. *Structure of lux-operon and corresponding enzymes (Prosser et al. 2000).*

Firefly luciferase have known to possess some advantages over bacterial luciferases. Generally, they are less sensitive to temperature, do not have substrate inhibition and able to provide a gene sequence not naturally present in bacteria with the possibility to choose form several light color options. However, insect luciferases require the presence of specific substrates united under the name of luciferin for light emission. Reaction is also ATP-dependent and goes in the presence of Mg^{2+} ions:

$$\text{D-Luciferin} + \text{ATP} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light} \qquad (3)$$

Usage of luminescence as a marker provides several benefits in comparison with fluorescence and conventional plating method. First of all, luminescence allows tracking the activity and its fluctuations in real time. In contrast to GFP, light is not accumulated in the cell and the detected intensity corresponds to the level of enzymatic activity and, respectively, the event marked by this activity at particular time moment. Secondly, there are no additional requirements, such as molecular excitation, for constructs with full *lux* operon. Finally, luminescence-based methods are more sensitive and allow detection of light very fast after the start of *lux*-genes expression. Moreover, use of luminescence helps to avoid excessive work and spending laboratory ware in comparison with conventional plating method. In addition to that, for the cultures in the exponential growth phase with constant expression of *lux*/*luc* genes it is fair to state that on the early stages, when luminescence levels are in the range of high resolution of utilized equip-

ment, the amount of emitted light is proportional to the biomass concentration. (Prosser et al. 2000)

Luminescence-based biomarker systems are reported to be utilized for a range of applications. These include tracking of fate of specific bacteria in environment, visualization of bacterial colonization of plant surfaces, whole-cell biosensors and so on. (Meighen 1991; Meighen 1993; Prosser et al. 2000)

Utilization of luminescence for the investigation of HGT have not yet received wide application. There are few studies focusing on conjugative transfer particularly in soil and connected biocenosis. One example is the research by Hoffmann and colleagues (1998) who suggested the gut of soil arthropod *Folsomia candida* able to provide favorable conditions for spread of plasmids to indigenous bacterial soil inhabitants. To examine this hypothesis the authors have constructed the range of plasmids, including self-transmissible and mobilizable, which were subsequently transformed in mobilizing and nonmobilizing *E. coli* strains. Both, firefly *luc* and bacterial *luxAB* luciferases were used as markers and were supposed to be constantly expressed either in donor or in recipient strains. In turn, the counter selection of donor cells was performed by inability of *E. coli* to grow on benzoic acid as a sole carbon source, while about 33 – 37 % of fecal bacteria possessed this feature. Authors have consciously sacrificed the fraction of indigenous bacteria unable to grow in the mentioned conditions since the aim of the study was to demonstrate that soil inhabitants can contribute to HGT rather than reliably count its rates or comprehensively explore the indigenous bacteria capable of receiving the plasmids. Nevertheless, transfer rates were estimated as well as obtained transconjugants were assigned to particular strains based on 16S rRNA analysis revealing that all the observed clones were the representatives of α, β and γ *Proteobacteria*. (Hoffmann et al. 1998)

In this study luminescence was employed not as a self-sufficient marker but in combination with antibiotic markers to distinguish between antibiotic resistant indigenous bacteria and resistant transconjugats. In addition to that, authors admit that at the end of experiment nearly all donor *E. coli* cells were digested in the gut of the arthropod, which means the release of free genetic material. Nonetheless, researchers have found that the possibility of natural plasmid transformation is negligible and tended to exclude such an event, since the study revealed that from all the combinations of self-transmissible and mobilizable plasmids with mobilizing and nonmobilizing strains the transfers have occurred only when transfer initiation genes were provided by the donor strain regardless the type of the plasmid. (Hoffmann et al. 1998)

Luminescence was also applied for the study of gene transfer in conditions mimicking environmental. Research have studied the conjugative plasmid transfer between introduced *E. coli*, *Pseudomonas putida* 2440 and indigenous soil bacteria in microcosms experiments (Palomares et al. 2001). Introduced strain carried luminescence genes on the transferrable plasmid, repressed in *E. coli* by chromosomally located repressor at usual cultivation temperatures and induced at higher temperatures. Researchers have employed eukaryotic luciferase genes *luc* (yellow-green) and *lucOR* (orange) for identi-

fication of transconjugants on plates with diluted bacterial samples cultured after co-incubation in soil microcosms. They demonstrated successful substitution of antibiotic markers by luminescence in experiments demonstrating HGT suggesting such a system as safe for field investigations. (Palomares et al. 2001) The use of such system, however, did not avoid plate cultivation and manual counting stage, which, as was already noted, limits the range of detectable transformants to readily culturable and tends to underestimate the total transformation events. In addition, authors utilized luminescence only for ease of identification of transformants after conventional surface cultivation, losing the advantage of possibility to observe transformation in real time.

The mentioned studies have employed luminescence method for observation of conjugation mode of HGT. In contrast to them, I will try to broaden luminescence application and use it for confirmation of natural transformation events. In addition, I will try to make benefit form advantages of luminescence method over other methods used for exploration of HGT and visualize gene acquisition in dynamic.

5 RESEARCH METHODS AND MATERIALS

This chapter provides information about bacterial strains and genetic constructs employed and designed for the purposes of this research. Additionally, it describes all laboratory protocols and materials utilized for experimental part of this study.

5.1 Bacterial strains and cultivation conditions

A. baylyi ADP1 (DSM 24193 strain), its derivative clones *A. baylyi* ADP1 3383 with knocked-out fatty acyl-CoA reductase *acr1* (ACIAD3383) and *A. baylyi* ADP1 3383 i/*luxAB*/CAM obtained in previous experiments by Suvi Myllyntausta, Tampere University of Technology molecular laboratory researcher, were used for experiments to be described further. All previously and newly constructed plasmids carrying target cassettes were cloned in *E. coli* XL1-Blue cells (Stratagene, USA).

All bacterial strains were cultivated in Luria-Bertani (LB) liquid or dense medium containing 1 g/L NaCl, supplemented with 1% glucose, 15 g/l agar for dense medium and corresponding antibiotic in concentration of 50 μg/ml (25 μg/ml for *E. coli*) where applicable. Liquid cultivations were grown aerobically at 30℃ (37℃ for *E. coli*), 300 rpm; surface cultivations were performed also aerobically at 30℃ incubator (37℃ for *E. coli*).

5.2 Gene constructs

All constructs utilized in this study were designed from previously reported SM100/pIX, i/pIX and j/pIX (Myllyntausta 2010). These vector templates (Fig. 4.1) contained the same plasmid backbone with chloramphenicol (CAM) resistance but differed in regions, which flank the cassette and are homologous to flanking regions of genes to be knocked-out. Thereby, three vector templates match three genes chosen *in A. baylyi* ADP1 genome for knock-outs by the corresponding cassettes. Each cassette contained *lac*/T5 constitutive promoter and multiple cloning site (MCS), which were the same for all the constructs. Additionally, each construct contained selection marker, which imparted CAM resistance in clones carrying i/pIX and j/pIX and kanamycin (KAN) resistance in clones with SM100/pIX. Each plasmid vector had the ability to be maintained in *E. coli* cells due to the presence of ColE1 region and lacked this ability in *A. baylyi* ADP1.

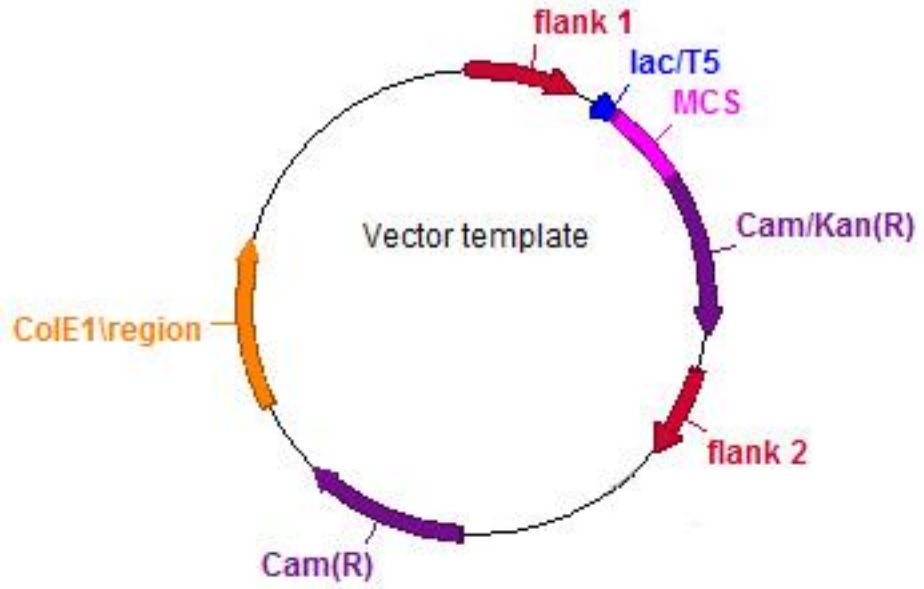

Figure 4.1. *General scheme of the vector templates used in this study. The cassette is confined within flanks 1 and 2 and contain lac/T5 promoter, MCS and CAM or KAN resistance gene. The rest, plasmid backbone, also includes CAM resistance gene and possesses ColE1 region for plasmid replication in E. coli.*

The general idea of functioning of the constructs was to receive the luminescence signal only when particular combination of *lux* genes is incorporated and expressed in *A. baylyi* ADP1 cell. These combinations are shown on Figure 4.2. In the first variant of combination, where *luxCD* genes responsible for the substrate synthesis are lacking, the luminescence production was supposed to be reached by means of addition of decanal substrate.

luxABE (or *luxA* + *luxBE*) + decanal → Luminescence

luxABE + *luxCD* → Luminescence

luxA + *luxBE* + *luxCD* → Luminescence

Figure 4.2. *General principle of lux-operon division between cassettes and production of luminescence signal as a consequence of incorporation of particular cassettes containing particular lux genes into A. baylyi ADP1 genome. For the ease of understanding information about the cassettes was omitted.*

Genes comprising bacterial lux-operon were amplified from Lux/pVKK81 plasmid (unpublished, Santala) with primers listed in Table 4.1. *LuxBE* gene was obtained as a ligation product of *Bam*HI-digested *luxB* and *luxE* genes, while *luxABE* gene – as a ligation product of *Bam*HI-digested *luxAB* and *luxE* genes. Here *LuxE* gene was amplified separately in order to damage the *Nde*I restriction site situated between *luxE* and *luxB* in

Lux/pVKK81 to make this site unique in the resulting construct. So, after amplification and ligation genes *luxA*, *luxBE*, *luxABE* and *luxCD* had unique *Nde*I and *Xho*I restriction sites, which were used to insert these genes into multiple cloning sites of template vectors. All the enzymes used in this study, including T4 DNA ligase and Phusion Hot Start (II) DNA Polymerase, and all the supplemented chemicals required for enzymatic reaction or amplification were produced by Fermentas, USA and were used according to standard protocols provided by manufacturer.

Table 4.1. *PCR primers used in study.*

Primer	**Sequence (5'-3')**	**Amplification target**	**Inserted restriction site**
ab1	ATATGGTACCTTGGA-TAACATGGTCCATTCATATC	SM100 Fwd	
ab12	CCTCGGCCCCCGAGGCCATGGGCGG TATGATGTAATACC	SM100 Rev	
ab34	AATACTCGAGTTAAGACAGA-GAAATTGCTTGATTTTC	*luxCD* Rev	
ab39	ATATCATATGAAATTT-GGAAACTTTTTGCTTAC	*luxA* Fwd	*Nde*I
ab57	ATATGGTACCCACACCAATTTTAG-CACCCGGAAAAAATG	i Fwd	
ab60	AA-TAGGCCCCCGAGGCCTGTCAAAA-GCATAGGAAGTGG	i Rev	
ab61	AATACCTAGGAAGAAGGAGA-TATACAT-ATGACTAAAAAAATTTCATTCATTA TTAAC	*luxCD* Fwd	
ab78	CAATCTCGAGTTAACTATCAAAC-GCTTCGGTTAAGC	*luxE* Rev	*Xho*I
ab105	CAATGGATCCTTATCTTGAGGAG-TAAAACAGGTATGACTAG-TTATGTTGATAAACAA-GAAATTACAG	*luxE* Fwd	*Bam*HI
ab107	ATATGGTACCGATGTCGAAC-GCTCAAACTGG	j Fwd	
ab110	CCTCGGCCCCCGAGGCCGTCCAAGT TAAAGTAAAAGAGGGTGATC	j Rev	
ab117	ATATCTCGAGTTAATATAA-TAGCGAACGTTGTTTTTC	*luxA* Rev	*Xho*I

Table 4.1. *Continued*

Primer	**Sequence (5'-3')**	**Amplification target**	**Inserted restriction site**
ab118	ATATCATATGAAATTT-GGATTGTTCTTCCTTAAC	*luxB* Fwd	*Nde*I
ab119	ATATGGAT-TCTTAGGTATATTCCATGTGG-TACTTCTTAATATTATC	*luxB* Rev	*Bam*HI
ab122	CAATGAATTCCCTAGGGACTG-GAAAGCGGGCAGTGAG	Universal cas-sette, Fwd	
ab123	CAATCTGCAGCAATT-GCAGGGCGCGTGGGGATC	Universal cas-sette, Rev	

Apart from insertions of *lux* genes into the MCSs of the cassettes some additional changes were made to optimize the work of the constructs in target application. So, CAM resistance gene located within the cassette in vector i/pIX/*luxABE* was replaced by KAN resistance gene from SM100/pIX vector by means of restriction of both marker genes with *Avr*II and *Xho*I enzymes, product separation by electrophoresis, purification of KAN gene and the remained part of i/pIX/*luxABE* plasmid with Gene Jet^{TM} Gel Extraction Kit (Fermentas, USA) and their ligation according to standard protocol (Fig 4.3). 2µl of mixture with heat-inactivated ligase was used for electroporation of *E. coli* XL-1 Blue competent cells according to standard protocol both with ligation and transformation controls. Positive clones were selected from LA-plates with 25 µg/ml KAN.

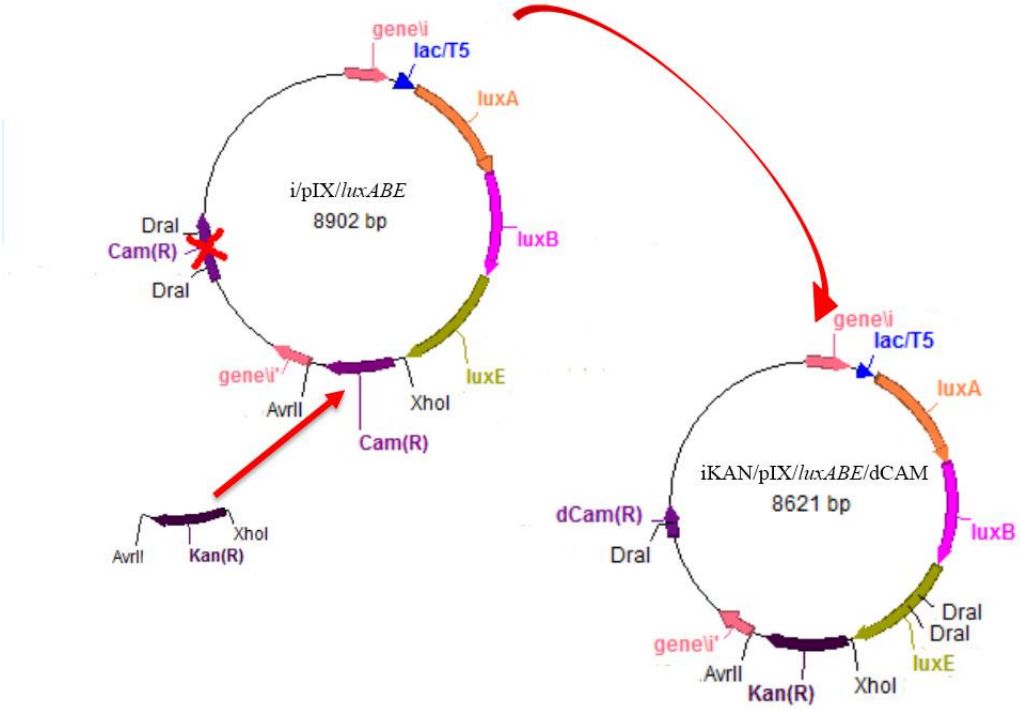

Figure 4.3. *Scheme of modification of i/pIX/luxABE to iKAN/pIX/luxABE/dCAM vector.*

iKAN/pIX/*luxABE* vector was also reconstructed to get rid of CAM resistance gene in the plasmid backbone (Fig. 4.3). For that j/pIX/*luxCD* vector was digested at unique *Kpn*I and *Sfi*I restriction sites located at the beginning and the end of cassette and with *Dra*I enzyme having seven restriction sites in the vector and only two of them not within the cassette, in CAM resistance gene in particular (Fig. 4.4). J/pIX/*luxCD* construct was chosen for this triple digestion because resulted eight digestion products were easily separable by size in gel electrophoresis. Among them there were two target pieces – beginning of the plasmid backbone confined by *Kpn*I sticky end and *Dra*I blunt end and ending of the plasmid confined by *Dra*I blunt end and *Sfi*I sticky end. These two products were purified and ligated according to standard ligation protocol for blunt ends. To increase the yield of right ligation product in mixture it was again digested by *Kpn*I and *Sfi*I enzymes after ligase inactivation and purification of mixture with Gene Jet^{TM} PCR Purification Kit (Fermentas, USA).

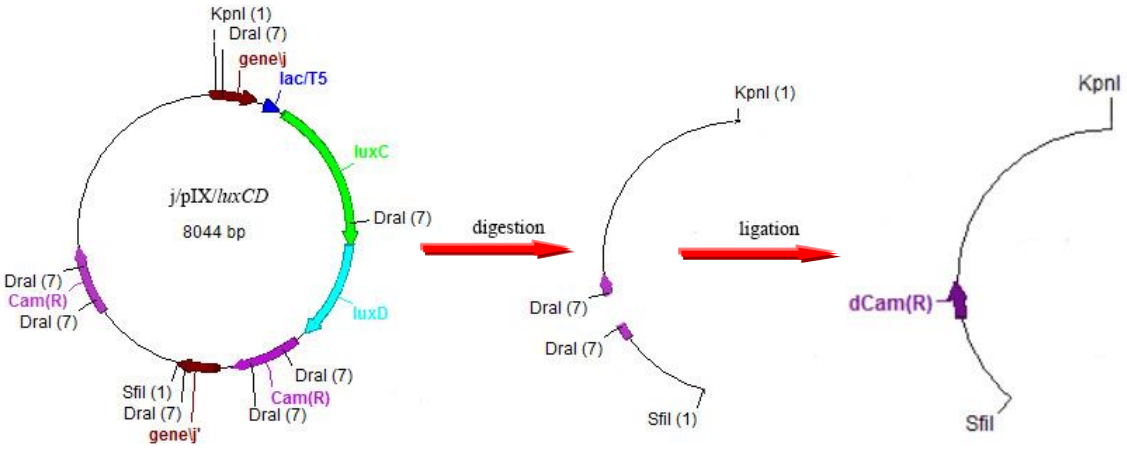

Figure 4.4. *Scheme of damaging of CAM resistance gene in the plasmid backbone by triple enzyme digestion of j/pIX/luxCD vector. The obtained backbone was subsequently ligated with all cassettes.*

Meanwhile separate portion of iKAN/pIX/*luxABE* was subjected to double digestion with only two *Kpn*I and *Sfi*I enzymes. Cassette part was purified and used for ligation with newly obtained plasmid backbone containing damaged CAM resistance gene (dCAM). 2µl of mixture with heat-inactivated ligase was used for transformation by electroporation of *E. coli* XL-1 Blue competent cells according to standard protocol with the following seeding of cells on LA-plates with 25 µg/ml KAN.

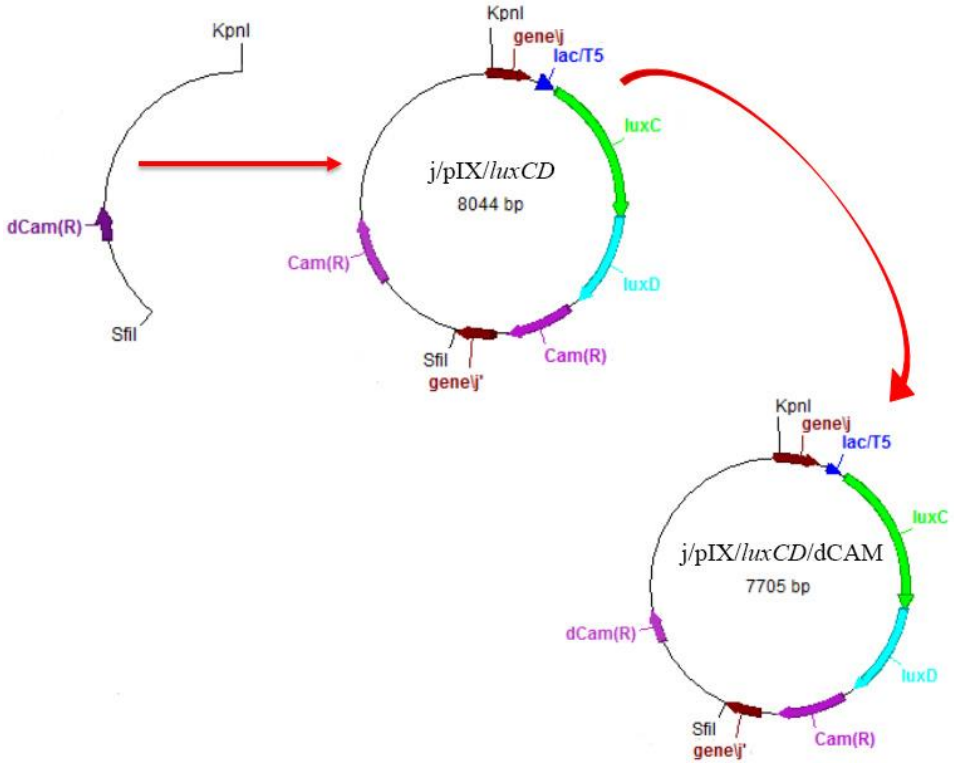

Figure 4.5. *Scheme of modification of j/pIX/luxCD to j/pIX/luxCD/dCAM vector.*

Seeding of all the selected clones on LA-plates containing 1) 25 µg/ml KAN and 2) 25 µg/ml KAN and 25 µg/ml CAM revealed that all clones are resistant to KAN and sensitive to CAM confirming the successful construction of the right plasmid. After this verification, the plasmid the backbone with dCAM gene was restricted from obtained and amplified in *E. coli* XL-1 Blue plasmid iKAN/pIX/*luxABE*/dCAM with unique *Kpn*I and *Sfi*I enzymes, purified and ligated with the purified j/*luxCD* cassette in advance restricted with the same enzymes from j/pIX/*luxCD* to obtain finally j/pIX/*luxCD*/dCAM (Fig. 4.5). 2µl of mixture with heat-inactivated ligase was used for electroporation of *E. coli* XL-1 Blue competent cells according to standard protocol, clones were selected from LA-plates with 25 µg/ml CAM.

Table 4.2 *Gene constructs obtained in the study.*

Vector	**Knock-out target**	**Resistance in cassette**	**Resistance in plasmid backbone**
SM100/pIX/*luxA*	2837	KAN	CAM
SM100/pIX/*luxABE*	2837	KAN	CAM
i/pIX/*luxABE*	3381	CAM	CAM
iKAN/pIX/*luxABE*	3381	KAN	CAM
iKAN/pIX/*luxABE*/dCAM	3381	KAN	-
i/pIX/*luxBE*	3381	CAM	CAM
j/pIX/*luxCD*	3309	CAM	CAM
j/pIX/*luxCD*/dCAM	3309	CAM	-

In total, eight vectors were designed and constructed for the purposes of this study. Table 4.2 contains the information about structure of these vectors, genes they aimed to knock-out in *A. baylyi* ADP1 and antibiotic resistance markers located in different parts of vectors.

5.3 Transformation of *A. baylyi* ADP1

Two sets of transformation experiments were performed during this study. The first one was necessary for confirmation of successful work of all the engineered constructs. To verify the cassettes all of them either in linear or in circular form were transformed into *A. baylyi* ADP1 according to the following general protocol:

1. *A. baylyi* ADP1 strain fresh cultivation with OD_{600}=0.05 was prepared from overnight cultivation and grown until the exponential phase and OD_{600}=0.7;
2. Culture at exponential growth phase was divided into two tubes of equal volume and corresponding DNA was added to the transformation sample in the amount of 1μg/ml;
3. Cultivations were left overnight. Clones were selected on LA-plates containing 50 μg/ml of the corresponding antibiotic.

The transformation of *A. baylyi* ADP1 was performed sequentially with the obtained cassettes according to lux-operon restoration scheme (Fig. 4.2). Each subsequent cassette was incorporated in separate experiment in the intermediate clones, for which the successful insertion of the previous cassettes was verified in advance. In total, 11 derivative clones of *A. baylyi* ADP1 (Table 5.1) and one clone of *A. baylyi* ADP1 3383 (T16) was obtained and analyzed by amplification of the corresponding constructs or initial genes with primers listed in Table 4.1 from genomic DNA samples extracted from each clone with Gene Jet™ Genomic DNA Extraction Kit (Fermentas, USA). In addition to that, transformant colonies were checked on luminescence production with Xenogen (Perkin Elmer, USA) by application of three drops of 0.46% decanal (92%, Sigma-Aldrich, Germany) solution on the Petri dish cap or without any substrate in all the cases where luminescence was supposed to be produced.

The second set of transformation experiments was aimed on observation of gene transfer and natural transformation in dynamic. These real time transformations of *A. baylyi* ADP1 and its derivative clones were performed according to procedure the first two steps of which coincide with the general transformation protocol mentioned above. The following steps were:

3. Luminescence at zero and following time points was measured in three replicates for each sample on white opaque-bottom 96-well microtiter plates with Plate CHAMELEON™ Microplate Reader (Hindex, Finland);
4. All other relevant manipulations were carried out: OD_{600} measurements and platings of transformation and control samples on LA-plates with the corresponding antibiotic. The total time of experiment, time between measurements and their set varied depending on experiment conditions and objectives.

In some experiments luminescence measurements were performed totally automatically. In that case incubation of *A. baylyi* ADP1 with transforming DNA was carried out directly on microtiter plates in Plate CHAMELEON™ Microplate Reader providing culture shaking and constant 30℃ temperature for growth between measurements.

5.4 HGT experiment protocol

Experiments with co-culturing of *A. baylyi* ADP1 (3383) iKAN/*luxABE* with *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM were performed according to the following general protocol:

1. Fresh cultivations with OD_{600}=0.05 were prepared for both strains (plus *E. coli* XL-1 Blue carrying no plasmid as a control) from overnight cultivations and grown until the time *A. baylyi* ADP1 reached the exponential phase and OD_{600}=0.7;
2. *A. baylyi* ADP1 culture at exponential growth phase was divided into eight tubes of equal volume (further referred as standard volume);
3. The OD_{600} of fresh *E. coli* XL-1 Blue cultures was measured and all four *E. coli* cultures, including the overnight cultures, were diluted with the fresh LB medium till the smallest measured OD_{600} to make the amount of cells in each sample equal;
4. Then each of the four diluted *E. coli* XL-1 Blue cultures in the amount equal to the standard volume was taken and centrifuged to separate the cells from supernatant. The pellets were consequently resuspended in fresh LB medium till the standard volume, so that two samples, with cells and supernatant, were obtained from each of four *E. coli* cultures (fresh and overnight *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM and fresh and overnight *E. coli* XL-1 Blue carrying no plasmid), giving eight samples in total;
5. Each of eight resulted *E. coli* samples was merged with eight *A. baylyi* ADP1 aliquots in a separate tube. OD_{600} of each co-culture was measured and confirmed to be equal with others. Tubes were incubated in shaker at 300 rpm and 30℃;
6. Luminescence at zero and following time points was measured in three replicates for each co-culture on white opaque-bottom 96-well microtiter plates with Plate CHAMELEON™ Microplate Reader;
7. All other relevant manipulations were carried out: OD_{600} measurements and platings of mixtures on LA-plates with 50 μg/ml KAN and 50 μg/ml CAM. The total time of experiment, time between measurements and their set varied depending on experiment conditions and objectives.

For the ease and quickness of work necessary for handling of fast-growing exponential cultures all calculations were made automatically by the specially written script (Appendix 1) which utilized OD_{600} of all four *E. coli* cultures and OD_{600} of *A. baylyi* ADP1 culture as the input giving as the output the volumes of initial cultures and fresh LB medium needed to obtain the dilutions described in the protocol. All plates were be-

ing checked on luminescence production with Xenogen within the following two days first without and then with addition of decanal solution to the Petri dish cap.

6 RESULTS

This section contains information about results of experimental part of the thesis. The chapters reflect the flow of experiments and discuss the results of all important intermediate experiments and decisions made about changes in the overall direction of research made during drawing of intermediate conclusions. Prior to the actual real time transformation experiments, all the obtained constructs were verified and separately examined on ability to transform the naturally competent *A. baylyi* ADP1 strain and ability to result in production of luminescence as a consequence of expression of *lux*-operon genes grouped on different cassettes.

6.1 Verification of constructs and clones

Sequential transformations were performed to verify obtained constructs, their ability to transform *A. baylyi* ADP1 by homologous recombination, knock-out the right gene and ability to be expressed with production of luminescence. Table 5.1 summarizes the data about *A. baylyi* ADP1 clones obtained during constructs verification.

Table 5.1. *A. baylyi ADP1 clones obtained in the study and respective incorporated genetic constructs. Green color corresponds to expected genetic changes; red color denotes transformation events with unexpected mechanism and combination of genes.*

Gene \ Clone	T1	T4, T6	T71	T9	T10	T11	T12	T13	T14	T15
i/*luxABE* cassette	+	+								
luxABE gene	+	+								
Initial 3381 gene	-	-								
j/*luxCD* cassette		-	+		-		+		+	+
luxCD gene		+	+		+		+		+	+
Initial 3309 gene		+	-		+		-		-	-
i/*luxBE* cassette				+	+					
luxBE gene				+	+					
Initial 3381 gene				-	-					
SM100/*luxA* cassette				-	-					
luxA gene				+	+					
Initial 2837 gene				-	-					
SM100/*luxABE* cassette						-				
luxABE gene						+				
Initial 2837 gene						+				
iKAN/*luxABE* cassette								+	+	+
luxABE gene								+	+	+
Initial 3381 gene								-	-	-

6.1.1 Trial transformation of *A. baylyi* ADP1 with circular and linearized forms of i/pIX/*luxABE* plasmid and separate i/*luxABE* cassette

To compare the transformation capabilities of three forms of DNA constructs (intact plasmid, linearized plasmid and cassette) the trial *A. baylyi* ADP1 transformation experiments were undertaken. Gradual rise of luminescence proving transformation was monitored in real time with use of decanal substrate. Conventional plating method with subsequent examination of light emission served to account for success of transformation.

Experiment confirmed the previous research data (Palmen et al. 1993) and showed that intact plasmid was the most efficient in transformation, followed by linearized plasmid exhibiting insignificant luminescence increase with increase of DNA exposure time and cassette demonstrating negligible light emission after 6 hours of cultivation (Table 5.2). Plating method was not that sensitive and resulted in 24 (6 hours) and 48 (overnight) colonies for intact plasmid, one (6 hours) and two (overnight) colonies for linearized plasmid and none (6 hours) and 10 (overnight) colonies for cassette.

Table 5.2. *Average luminescence levels of liquid transformation mixtures, each measured at triple repeats at different time points after addition of different forms of transforming DNA.*

Time point / Construct	0 hours	6 hours	overnight cultivation
Intact i/pIX/*luxABE*	19±7	1695±110	6530±697
Linearized i/pIX/*luxABE*	9±7	231±45	2123±246
i/*luxABE* cassette	17±9	77±12	584±16
A. baylyi ADP1 control	33±15	11±5	15±7

Despite higher levels of luminescence in liquid cultivations, not all clones resulted from transformations with two forms of plasmid emitted light in the presence of decanal. This was suggested to be the consequence of possible appearance of "wrong" clones, in which not the cassette but the plasmid backbone confined within flanking region(s) and also containing CAM resistance marker was integrated into genome and resulted in the background on the plates. Therefore, to avoid this background, cassettes were offered for further experiments in spite of not so rapid growth of luminescence in liquid cultivation.

6.1.2 Sequential transformation of *A. baylyi* ADP1 with i/*luxABE* and j/*luxCD* cassettes

A. baylyi ADP1 T1 obtained from the previous experiment and containing only *luxABE* part of *lux*-operon successfully produced luminescence when decanal was added. How-

ever, without decanal T1 clone also produced significant background luminescence, which can be attributed to intrinsic production of aldehyde able to be a substrate for luciferase. Presence of *luxE* gene in the incorporated cassette can also play the role in background formation. This enzyme takes part in conversion of fatty acids to long chain aldehydes. In *A. baylyi* ADP1 there is a natural analog of *luxC* gene – *acr1* encoding fatty acid reductase, which can partially compensate the function of *luxC*. In this case, two of three functions of fatty acid reductase complex are already present in the cell, which can contribute to the substrate synthesis.

When the whole *lux*-operon was incorporated to the *A. baylyi* ADP1 genome by i/*luxABE* and j/*luxCD* cassettes, resulting in T4 and T6 clones, the luminescence, as expected, was successfully produced at a high level without any additional substrate. Despite this, both T4 and T6 clones were proven to contain 3309 gene, which had to be knocked-out by j-cassette, and only the part of j/*luxCD* cassette including *luxCD* gene incorporated into genome in some other place then it was expected. In contrast, j/*luxCD* cassette in clone T71 obtained by *A. baylyi* ADP1 transformation with j/pIX/*luxCD*, successfully knocked-out 3309 gene, confirming ability of construct to work as intended in addition to unexpected transformation events, which nonetheless also resulted in luminescent clones (Table 5.1).

6.1.3 Sequential transformation of *A. baylyi* ADP1 with i/*luxBE*, SM100/*luxA* and j/*luxCD* cassettes

This order of transformation was chosen for easier selection of transformants and monitoring of luminescence production. It was supposed to be easy to perform selection of i/*luxBE* positive clones on a basis of CAM resistance, then i/*luxBE* and SM100/*luxA* positive clones – on a basis of resistance to KAN and luminescence production in presence of decanal; and selection of clones with all three constructs on a basis of luminescence production without decanal addition.

Nevertheless, *A. baylyi* ADP1 sequential transformation with all three cassettes did not result in luminescent clones. In this three-step transformation integration of SM100/*luxA* cassette was found to be the first bottleneck. This construct had quite low transformation efficiency. Moreover, the majority of resulted clones were cassette-negative and did not produce or produced very little luminescence despite the presence of *luxA* gene incorporated into genome by some other mechanism than expected homologous recombination with target for knock-out gene.

The second bottleneck, observed both in transformations with two and especially with three cassettes, was the lack of selection markers. Original i- and j-cassettes both had the same CAM resistance marker. Initially luminescence was supposed to be the third marker enabling the counting of right colonies on the plates. However, on practice it appeared to be almost impossible and inaccurate accounting. In case if luminescence is utilized as the sole selection marker the chances to observe the transformants are de-

termined by a range of factors including their ability to grow competitively on the used media.

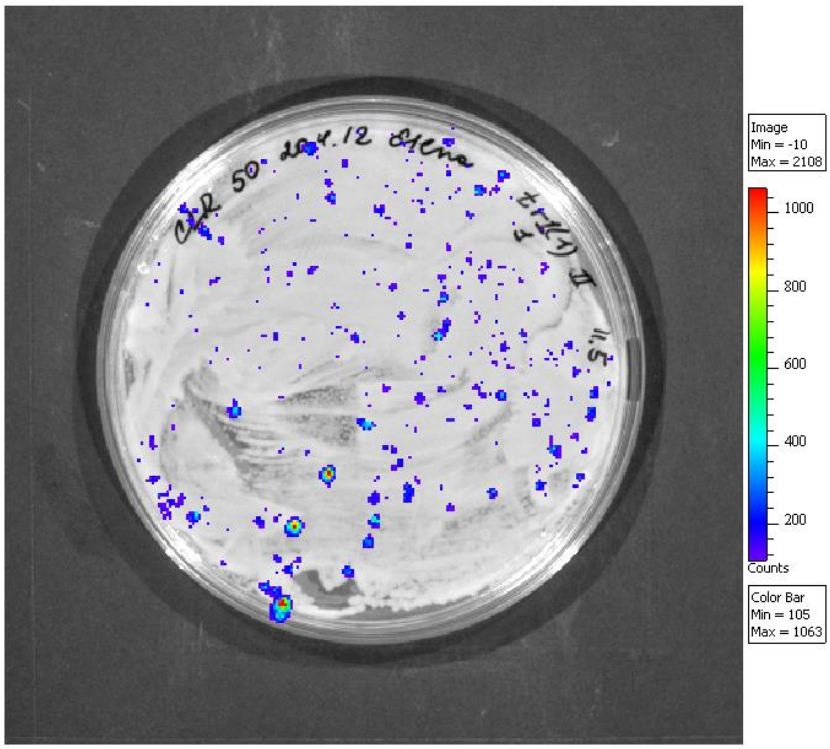

Figure 5.1. *Transformation of A. baylyi ADP1 already containing i/luxABE cassette (CAM) with j/luxCD cassette (CAM). Due to the same resistance marker in j/luxCD cassette it is impossible to reliably count transformed clones basing only on luminescence.*

In the last third experiment cells lacking the last third construct and carrying less "load" probably overgrew the right clones, which resulted in minor luminescent ripple on the plates without ability to count single colonies (Fig. 5.1), or in total absence of any signal even on plates with diluted cultivations. This confused luminescent ripple can also possibly be attributed to the background luminescence mentioned in previous section. Dilutions to the higher extent would give false average number of positive clones due to their relative fewness in comparison with untransformed cells. The obvious solution to this problem was the usage of distinct resistance markers ensuring the selective growth of only target clones. Therefore, the decision had been made to simplify the system towards the use of only two constructs with different resistance markers, namely, SM100/*luxABE* (KAN) and j/*luxCD* (CAM).

6.1.4 Sequential transformation of *A. baylyi* ADP1 with SM100/*luxABE* and j/*luxCD* cassettes or plasmids

Since cassette form of DNA was used for transformations, for this set of experiments it was also required to obtain SM100/*luxABE* cassette first. Amplification with ab1 and ab12 primers was very poor and insufficient for transformation purposes, while amplifi-

cation with ab122 and ab123 primers, universal for all cassettes, gave amplification product of size less than expected. This product was examined for transformation of parent *A. baylyi* ADP1 strain and T71 clone already containing j/*luxCD* cassette but this did not lead to any luminescent colonies. Therefore, restricted from the SM100/pIX/*luxABE* (KAN) vector SM100/*luxABE* cassette of the right size along with the vector itself was also tested on ability to transform the same strains. Transformation with the cassette also did not yield any colonies, while plates with cells transformed with intact vector contained only two clones exhibiting very low luminescence level. Both of them (named as T11) were proved to contain initial 2837 gene instead of cassette and *luxABE* gene incorporated into genome again in some other place than expected. For these reasons, SM100/pIX/*luxABE* construct was admitted to be inefficient to be used in this study.

Following conclusion had been drawn: constructs that could be successfully used in this study should be efficient, enable good selection of clones, i.e. contain distinct markers, and should not result in background colonies. From all experiments described above it is clear that i- and j-constructs are the most efficient and are the best two candidates. Further, to facilitate study purposes in the next transformation experiments it was decided to utilize intact plasmids, which resulted in more dynamic increase of luminescence in time as was demonstrated in section 5.1.1. In order to be able to use plasmids instead of cassettes it is essential to get rid of the same resistance marker into the plasmid backbone to avoid the background colonies. As a consequence, the following changes were introduced to i/pIX/*luxABE* and j/pIX/*luxCD* vectors: 1) KAN^R gene replaced CAM^R gene in i/*luxABE* cassette; 2) CAM^R gene in the backbone of both vectors was damaged and resulted $dCAM^R$ gene part, which was proved not to impart cells with resistance to chloramphenicol.

6.1.5 Sequential transformation of *A. baylyi* ADP1 with iKAN/pIX/*luxABE*/dCAM and j/pIX/*luxCD*/dCAM plasmids

Modified vectors named as iKAN/pIX/*luxABE*/dCAM and j/pIX/*luxCD*/dCAM were tested in the same kind of experiments as previous constructs. Sequential transformation was efficient in both forward and reverse order. Selection of final i- and j-cassettes positive clones on plates with both antibiotics and their consequent examination on luminescence confirmed that there is no background colonies and constructs work in intended manner.

It was also noted that colonies containing both constructs partially lose ability to emit light on the second day of surface cultivation. However, when decanal was added all colonies produced relatively high level of luminescence proving synthesis of active luciferase. This phenomenon is most probably connected with changes in metabolism on different growth stages. Bacteria at exponential growth phase have the active supply of precursors for synthesis of the aldehyde substrate, in absence of which the “dark decay” takes place, as well as active regeneration of cofactors such as reduced flavin

mononucleotide $FMNH_2$ essential for bioluminescence reaction and, as previously noted, able to be a bottleneck in light production.

6.2 Transformation experiments tracked in real time

This subchapter describes the application of developed system to the actual experiments on tracking natural transformation and HGT dynamics in real time. First, experiments on visualizing of natural transformation in *A. baylyi* ADP1 with each of designed constructs are discussed followed by simultaneous two-cassette transformation. The subchapter also describes the results obtained in experiments comparing the time frames of natural plasmid transformation and natural transformation with subsequent homologous recombination. Finally, application of system for studying of HGT between *A. baylyi* ADP1 and *E. coli* XL-1 Blue is reviewed. All experiments were performed either automatically with programmed CHAMELEON™ Microplate Reader or manually with examination of culture aliquots taken from cultivation tubes at each time point.

6.2.1 Transformation of *A. baylyi* ADP1 with iKAN/pIX/*luxABE*/dCAM

Transformation tests with new iKAN/pIX/*luxABE*/dCAM vector demonstrated that liquid transformation mixture almost did not exhibit increase of luminescence with time (Fig. 5.2). On the other hand, it was possible to assert that the transformation was successful since selective plates contained colonies luminescent with decanal (Fig. 5.3).

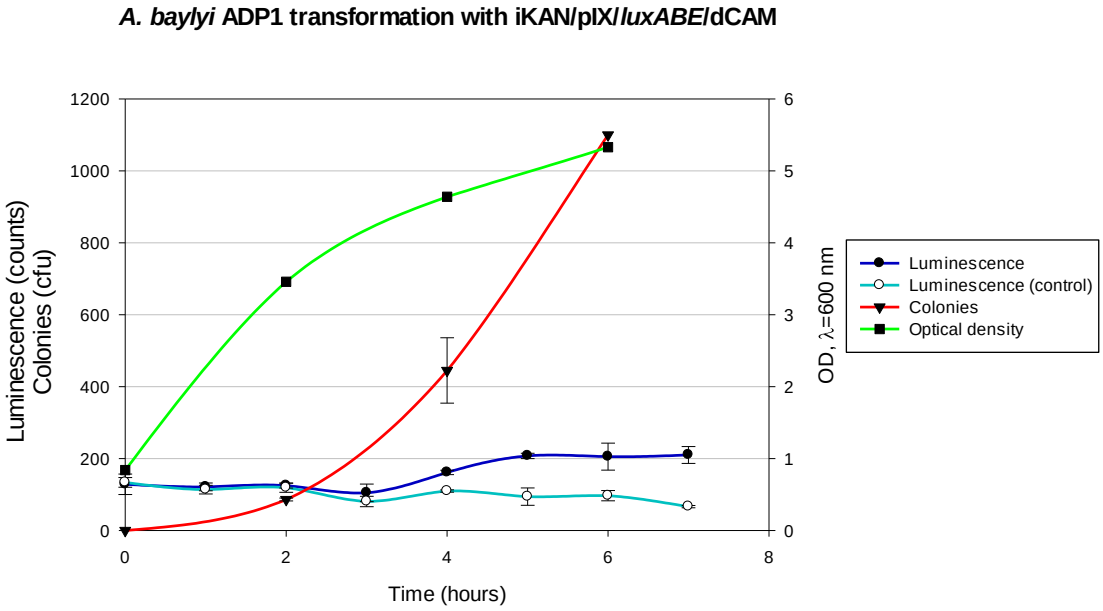

Figure 5.2. *Transformation of A. baylyi ADP1 with iKAN/pIX/luxABE/dCAM construct. Optical density of cultivation and amount of positive transformants on plates increase, while luminescence of transformation mixture is close to that of the control. Vertical bars present standard deviations.*

The opposite situation was observed when cells were transformed with old i/pIX/*luxABE* construct. Significant increase of luminescence proving the transformation was detectable already starting from the 3rd hour of cultivation, while, for some reason, it was impossible to get any luminescent clones on plates (Fig. 5.4).

iKAN/pIX/*luxABE* intermediate construct was also examined to check the possible effect of dCAM^R gene product and showed the same luminescence profile as iKAN/pIX/*luxABE*/dCAM confirming no difference. In addition to that, to exclude mutations in regulation sequences and genes responsible for luminescence the beginning of i-cassette of iKAN/pIX/*luxABE*/dCAM vector was restricted with *Kpn*I and *Xho*I enzymes. Resulting part containing *i*-flanking gene, promoter and *luxABE* gene was replaced by the corresponding piece from i/pIX/*luxABE* vector. However, newly obtained iKAN/pIX/*luxABE*/dCAM vector behaved in the same manner as the previous one.

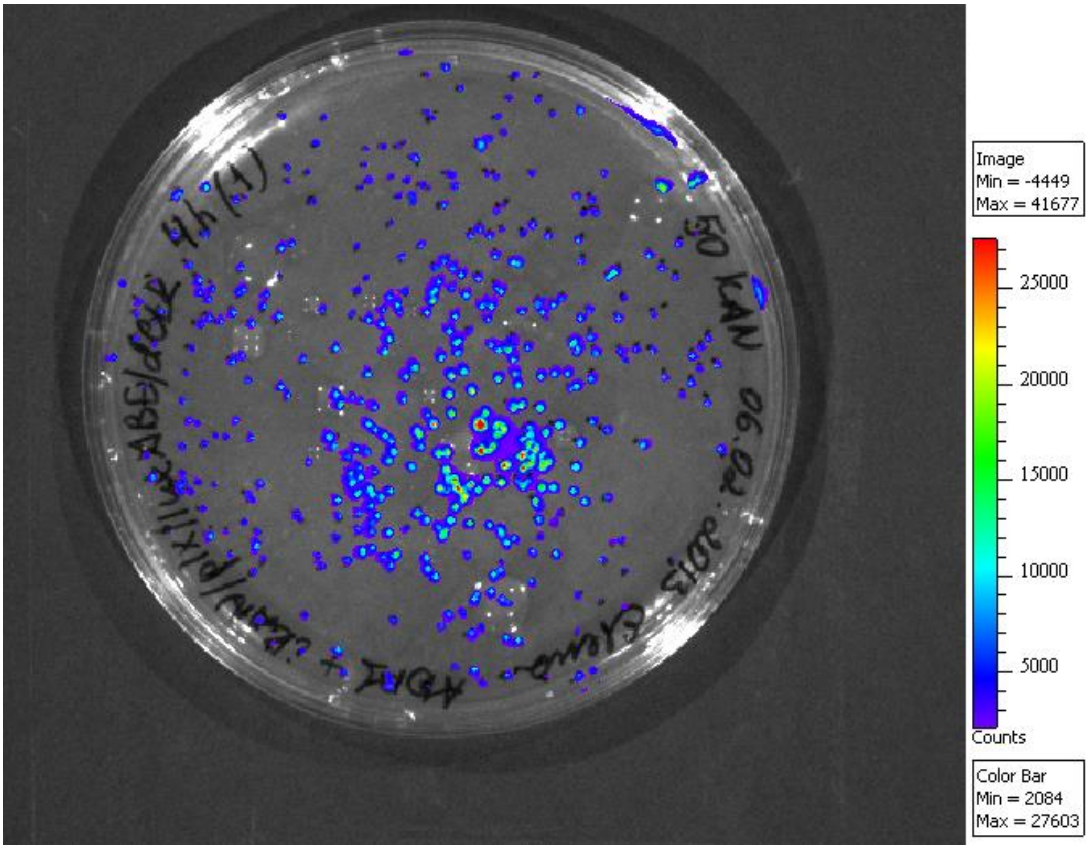

Figure 5.3. *Transformation of A. baylyi ADP1 with iKAN/pIX/luxABE/dCAM construct. Selective plate seeded after 4 hours after addition of DNA contains many transformants luminescent with decanal.*

These facts force to draw the conclusion about the role of CAM^R and KAN^R markers in work of i-cassette. CAM^R marker particularly for i-vector makes selection of clones by conventional plating method inefficient, whilst allows tracking of luminescence growth in time, i.e. to follow the transformation dynamics. KAN^R marker perfectly works for clones' selection but liquid cultivations of transformed cells lose the ability to produce light during transformation, thereby, doing the whole construct inefficient in studying the natural transformation with luminescence.

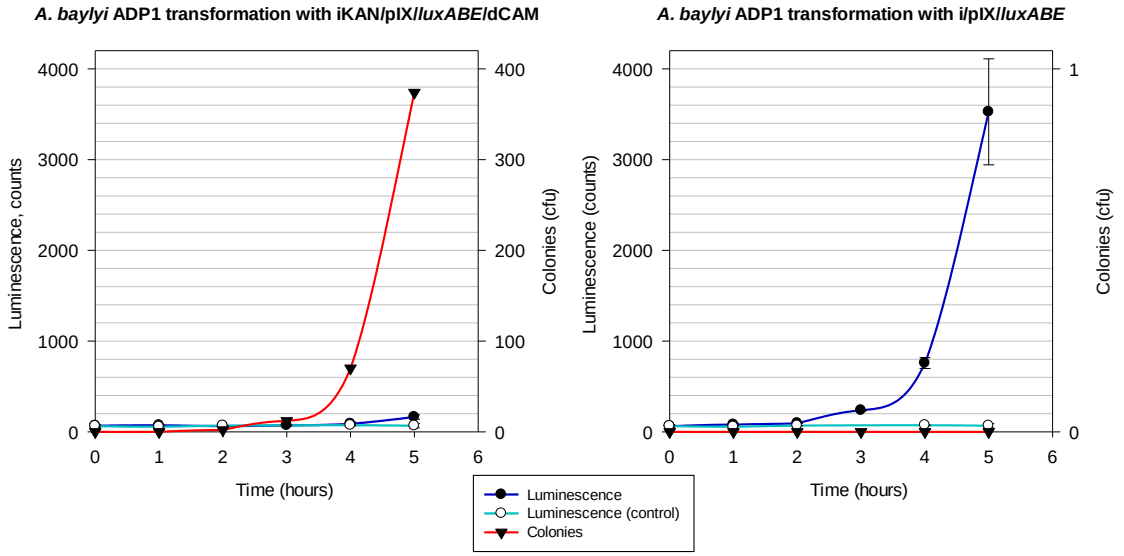

Figure 5.4. *Comparative test on transformation of A. baylyi ADP1 with iKAN/pIX/luxABE/dCAM (left) and i/pIX/luxABE (right). Vertical bars present standard deviations.*

Nevertheless, when selection pressure was applied to these liquid transformation mixtures (i.e. kanamycin was added), luminescence level started to increase gradually. On the one hand, it may indicate that transformation with these two constructs happens in the same rate but cells containing iKAN/*luxABE* cassette are generally less luminescent that cells with i/*luxABE* cassette, which affects the overall luminescence level. On the other hand, it is also possible to assume that iKAN/*luxABE* cassette is incorporated into genome less often than i/*luxABE* cassette resulting in less clones and, hence, lower luminescence. However, since CAM^R and KAN^R markers work in plating method with different efficiency for the same transformation, it is impossible to compare the abundance of positive clones in cultivations and say which hypothesis is closer to reality. Due to the same reason, it is also inaccurate to suggest looking at Figure 5.4 that plating method and luminescence tracking method are equally sensitive only because in the first experiment colonies appeared on plates starting from the second hour and in the parallel experiment luminescence began to rise at the same time point. The only obvious conclusion that can be drawn is that plating method was more sensitive for detecting the events of transformation with iKAN/*luxABE* cassette, while luminescence tracking demonstrated the presence of cells transformed with i/*luxABE* cassette where selective plating appeared to be inefficient.

6.2.2 Transformation of *A. baylyi* ADP1 T13 with j/pIX/*luxCD*/dCAM

The real time transformation experiments with j/pIX/*luxCD*/dCAM construct performed in the automatic mode confirmed that the construct functions properly. *A. baylyi* ADP1 T13 clone obtained in the previous experiment and already containing iKAN/*luxABE* cassette initially exhibited approximately 20000 counts luminescence background,

which, nonetheless, did not hinder the tracking of transformation because of the high transformation efficiency of construct and fast growing luminescence signal produced by transformants (Fig. 5.5). This proved the successful work of the second part of designed system and positive correlation between the rise of light emission and increase of number of clones producing this light estimated with the conventional plating method.

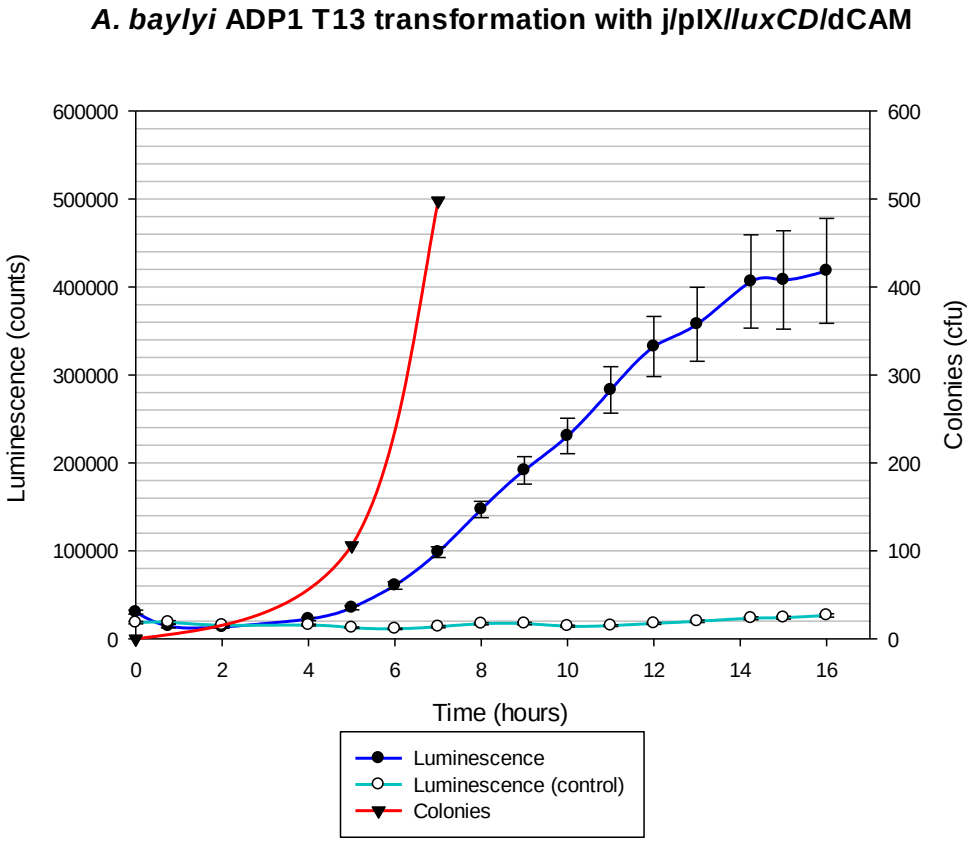

Figure 5.5. *Transformation of A. baylyi ADP1 T13 already containing iKAN/luxABE cassette in genome with j/pIX/luxCD/dCAM vector. Luminescence curves indicate light emission obtained from 200 µl of corresponding mixture at different time points. The red curve shows the amount of colonies on selective plates with 100 µl of transformation mixture seeded at different time points. Vertical bars present standard deviations.*

6.2.3 Transformation of *A. baylyi* ADP1 3383 KAN/*luxABE* with j/pIX/*luxCD*/dCAM and pBAV1C *ara luxCD* (CAM)

In tubes at proper shaking the transformation process, obviously, proceeds quicker than on plates because of more intensive culture growth. That was confirmed by the next experiment results indicated on Figure 5.6. This test was supposed to demonstrate the difference in time between transformation and subsequent recombination event, which takes place during *A. baylyi* transformation with j/pIX/*luxCD*/dCAM, and solely transformation event, for which pBAV1C *ara luxCD* broad host plasmid induced by L-arabinose was employed.

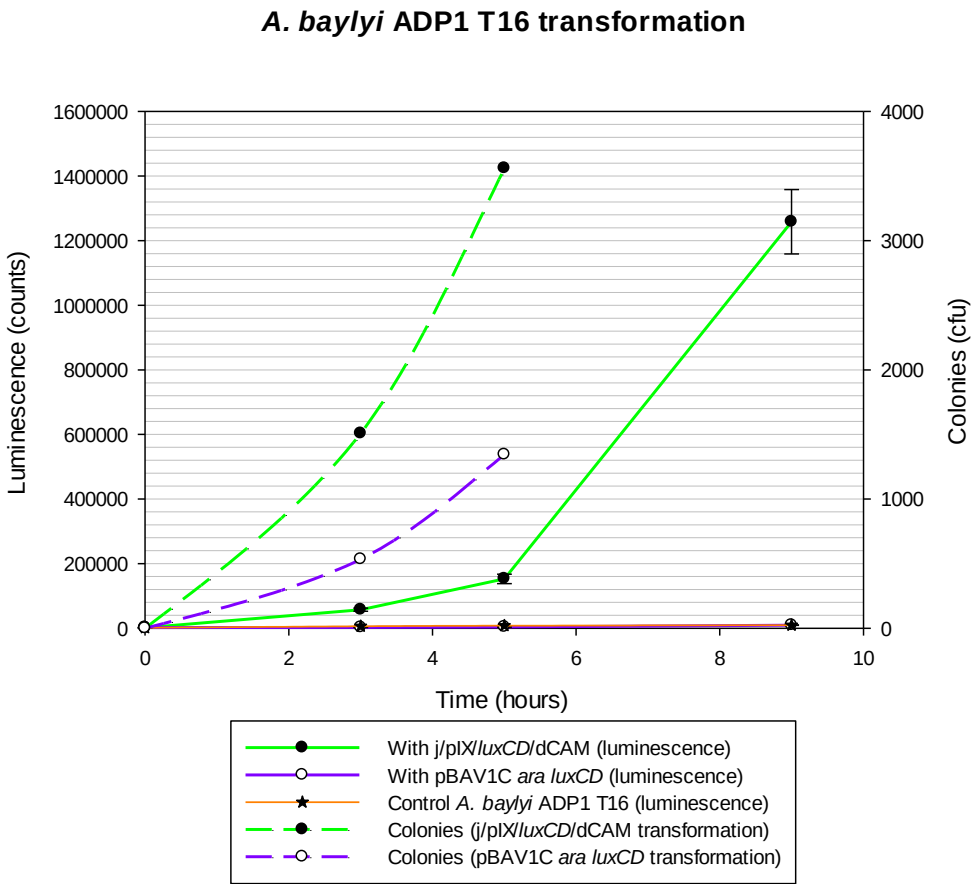

Figure 5.6. *Transformations of A. baylyi ADP1 3383 iKAN/luxABE (referred as T16) with j/pIX/luxCD/dCAM and pBAV1C ara luxCD plasmid. Vertical bars present standard deviations.*

In the trial experiments on transformation of *A. baylyi* ADP1 T13 with pBAV1C *ara luxCD(E)* it was difficult to differentiate between possible luminescence signal coming from transformed cells and background signal intrinsic to T13 clone. Therefore, *A. baylyi* ADP1 T16 clone containing *luxABE* genes was obtained from *A. baylyi* ADP1 3383 mutant (KAN) with knocked-out fatty acyl-CoA reductase *acr1* (ACIAD3383), which can contribute to aldehyde production and, hence, to formation of background signal. During *A. baylyi* ADP1 3383 transformation with iKAN/pIX/*luxABE*/dCAM several clones were chosen but, due to the difficulties with selection basing on only luminescence (as transformed strain already contains KAN^R), they all proved to have *luxABE* gene not within iKAN/*luxABE* cassette in expected place but in some other unknown location in genome. This can be the possible consequence of alternative KAN^R homology region presence in the host genome and subsequent recombination of vector part using this homology. Nevertheless, all clones were able to produce high level of luminescence and particularly T16 clone was proven to produce less background luminescence in the absence of substrate and more luminescence when substrate was present in comparison with other clones.

However, as Figure 5.6 shows, it was still impossible to reach detectable luminescence increase form pBAV1C *ara luxCD* transformation mixture despite the presence of

luminescent transformants on CAM, 1% L-arabinose selection plates. Therefore, it was difficult to compare the transformation on a basis of lux-operon expression. Ideally, to be able to compare these events using luminescence production it would be logically to create the similar expression conditions for *lux*-operon genes in cassettes and plasmid, i.e. to place them at least under the same T5 promoter. Of course, this would not cover the differences between expression from plasmid and from genome, but probably help to avoid the induction problems faced on practice in this experiment. As for conventional plating method, surprisingly, it showed that transformation and recombination with j/pIX/*luxCD*/dCAM construct happened faster than just transformation with pBAV1C *ara luxCD,* which is reflected in the amount of positive clones on selective plates.

6.2.4 Simultaneous transformation of *A. baylyi* ADP1 3383 with i/pIX/*luxABE* and j/pIX/*luxCD*/dCAM

For experiments with simultaneous transformation with two constructs *A. baylyi* ADP1 3383 was chosen since, as in previous experiment, it was expected to produce lower luminescence background. In addition to that, as the current study had the goal to demonstrate the natural transformation in dynamic in real time with use of luminescence, i/pIX/*luxABE* was preferred over iKAN/pIX/*luxABE*/dCAM, which did not provide the transformants with possibility to produce light in liquid transformation mixtures (section 5.2.1) . However, such choice made it impossible to correlate the light emission increase with amount of positive clones on plates since both transformable constructs possess CAM^R as a selection marker. Therefore, since there were no plating part in this experiment, it was carried out by CHAMELEON in an automatic mode to have a benefit of making more frequent luminescence measurements.

As it was demonstrated in the previous sections, j/pIX/*luxCD*/dCAM had much higher transformation efficiency than any of i-constructs confirming successful transformation already on 3^{rd} hour of cultivation with more than 10-times difference in luminescence in comparison to control sample, while for i/pIX/*luxABE* the same ratio was observed only after 4^{th} hour. Figure 5.7 evidences that the event of double transformation of the same cell obviously happened less often than transformation with the single construct and some difference between luminescence levels of transformation and control mixtures started to be notable only after 8 hours of cultivation and with the maximum 3-fold luminescence difference as well as very low overall luminescence level of 200 counts. Such a low signal corresponding to the low amount of full *lux*-operon positive clones can also partially be explained by possible substitution of one cassette already in genome by other cassette or its part through homologous recombination since both cassettes contained CAM^R gene. Moreover, simultaneous presence of two constructs in mixture had to reduce the transformation frequency of each separate construct, since, according to the literature data, DNA competes for the finite amount of DNA binding sites in cell (Palmen et al. 1993). Also, the growth conditions during such an

automated cultivation in CHAMELEON were generally less favored by cells in this and other experiments than the usual cultivation in shaker and this can be readily seen on Figure 5.7 as a delayed beginning of luminescence production starting from the 8th hour.

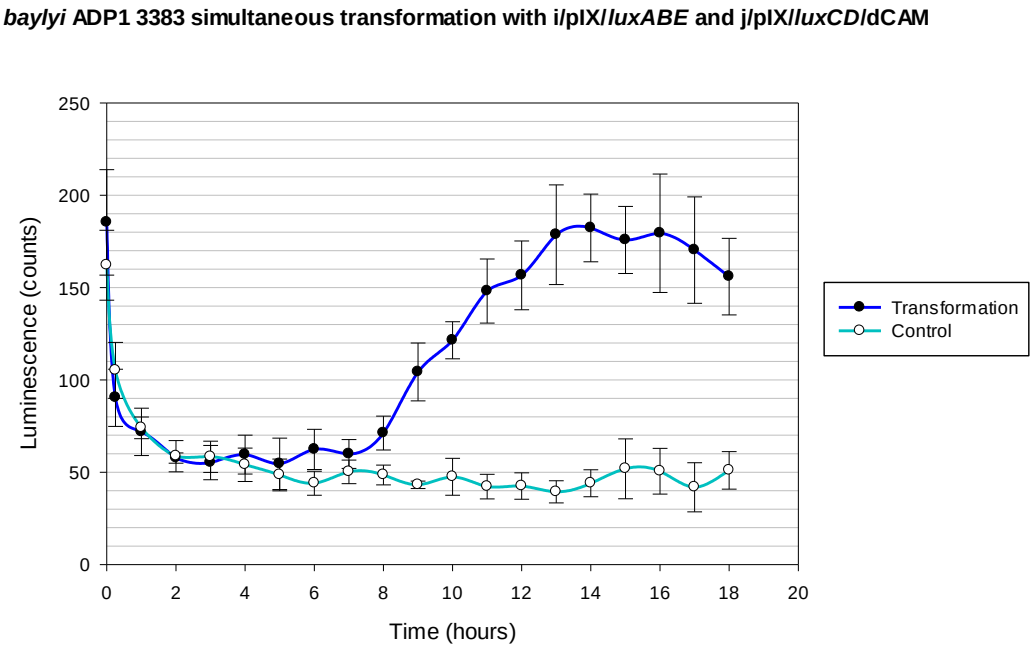

Figure 5.7. *Transformation of A. baylyi ADP1 3383 with i/pIX/luxABE (CAM) and j/pIX/luxCD/dCAM vectors at the same time. Vertical bars present standard deviations.*

In addition, some may argue that observed increase of luminescence signal could be connected with the background luminescence of clones containing i/*luxABE*-cassette alone rather than both cassettes. However, the liquid monoculture of *A. baylyi* ADP1 T16 produced luminescence only at the level of 10^4 counts and it seems unlikely that only few *luxABE* positive clones were able to produce the distinguishable background signal. Hence, from an overall perspective, two cassettes transformation events at single transformation experiment were quite rare, but detectable with the current system in real time with use of luminescence.

6.3 Horizontal gene transfer experiments

The developed system was further used to explore the HGT from *E. coli* XL-1 Blue containing j/pIX/*luxCD*/dCAM plasmid to low background *A. baylyi* ADP1 T16 clone with iKAN/*luxABE* cassette in the genome. For that, these two strains were co-cultured in the same tube at conditions beneficial for *A. baylyi* being the bacteria supposed to be the DNA recipient and producer of luminescence upon collection of complete *lux*-operon within cells. For the more accurate consideration of the T16 background luminescence the control samples were also set as co-culture of T16 clone with *E. coli* XL-1 Blue strain containing no plasmid of interest to account for natural space and nutrition competition processes between two distinct bacterial lineages. Overnight and fresh cul-

tivations of *E. coli* cells were used for parallel co-culturing to take into account possible differences between DNA donors in distinct growth phases.

From figure 5.8 it is clear that *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM overnight culture had much higher capacity towards horizontal gene transfer than fresh culture, which was also confirmed by the platings. Since the overall amount of *E. coli* cells added to the co-culturing mixtures was the same, it was logically to test further whether this difference in transformation frequency came from free DNA released from the cell debris, which was obviously present in higher amounts in the overnight cultivation. Therefore, the next experiment split into two parts, namely, co-culturing with *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM centrifuged and resuspended into the fresh medium and co-culturing with supernatant obtained during the centrifugation of these cells.

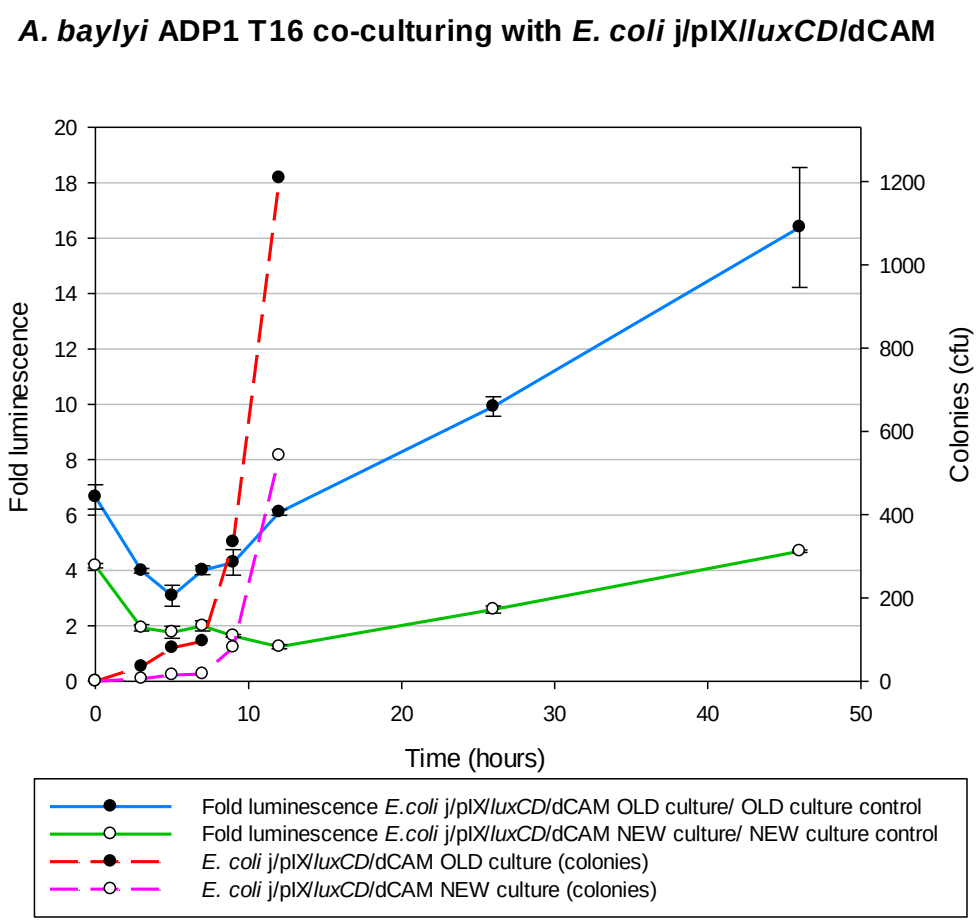

Figure 5.8. *Examination of the possibility of HGT between E. coli XL-1 Blue j/pIX/luxCD/dCAM and A. baylyi ADP1 T16. Vertical bars present standard deviations.*

As figure 5.9 shows, in experiments with resuspended cells the levels of fold-luminescence for both overnight and fresh *E. coli* co-cultivations, calculated as the ratio of luminescence in the actual co-culturing mixture to luminescence in the corresponding control mixture at particular time point, were comparable for fresh and overnight cultures, as well as the corresponding amounts of colonies. Hence, most probably, in previous experiment cells did not contribute much to the raise of luminescence, that is the massive transformation happened not due to the direct HGT through cell-to-cell interac-

tion. At the same time, experiments with supernatant confirmed that transformed *A. baylyi* appeared with the highest frequency in the overnight co-culturing mixture, which was evidenced with both luminescence and plating assays. Therefore, the conclusion can be drawn about the key role of DNA release from the dead cells and its uptake by *A. baylyi* ADP1 cells as the main mechanism of HGT in this co-culturing experiment.

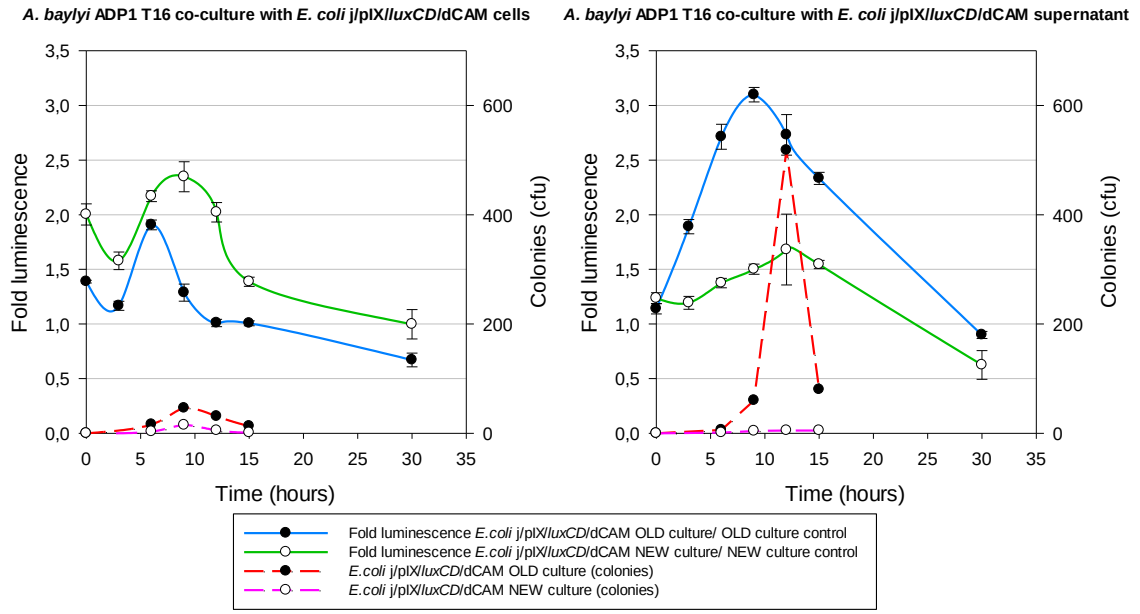

Figure 5.9. *Experiments with co-culturing of A. baylyi ADP1 T16 with: left – centrifuged and resuspended in the fresh medium E. coli XL-1 Blue j/pIX/luxCD/dCAM cells; right – supernatant obtained from centrifugation of E. coli XL-1 Blue j/pIX/luxCD/dCAM culture. Vertical bars present standard deviations.*

These two experiments also revealed that even in the beginning of test the significant difference was observed in luminescence levels between the actual transformation and the control mixtures (Fig. 5.8). At zero time point the mixture prepared from old *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM was seven times more luminescent than its corresponding control, for fresh *E. coli* cultivation mixture it reached four-fold difference. This fact suggests several possible scenarios: 1) the release of luciferase synthesized by *A. baylyi* ADP1 T16 into medium and its further uptake by *E. coli* cells producing substrate; 2) release of the substrate or enzymes responsible for substrate production produced in *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM and their subsequent penetration in *A. baylyi* cells; 3) or release of both products and extracellular luminescence reaction. Moreover, within the first five hours this ratio decreased, which could possibly be the evidence of substrate depletion in the transformation mixture, while further increase could be attributed to the transformation events and luminescence production already in *A. baylyi* cells harboring the whole *lux*-operon.

The presence of luciferase enzyme in the cultivation broth could be tested with addition of decanal to the filtered *A. baylyi* ADP1 T16 supernatant. The same kind of assay with luciferase substrate produced in *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM would be more problematic to perform due to the high price of purified luciferase. However, co-

culturing of *A. baylyi* ADP1 T16 with *E. coli* XL-1 Blue j/pIX/*luxCD*/dCAM supernatant demonstrated that initial luminescence levels of transformation mixtures almost coincided with control mixture luminescence at zero time point, thereby declining the hypothesis about ready aldehyde substrate presence into the extracellular space (Fig. 5.9).

7 DISCUSSION

7.1 Verification of constructs and clones

The work proved that there are multiple factors influencing the natural transformation of *A. baylyi* ADP1. J-, i- and SM100-constructs tested in this study transformed *A. baylyi* not only with different efficiencies, which mean the strict dependence on the knock-out gene accessibility, but resulting in clones with similar genetic changes but varying level of luminescence. For some transformations, constructs worked not in an expected manner, being incorporated and expressed from some other genome positions than it was initially intended. In case like this it is hard to predict the level of cassette's expression since it is impossible to say definitely what regulation sequences are situated upstream the incorporated gene. Moreover, it is difficult to say how many copies of the target gene the luminescent clone has because of the random incorporation events. This obviously also affected the luminescence level of particular clones and, hence, the overall liquid culture light emission. Such uncertainties significantly interfered with the initial purpose of the developed system, i.e. with correlation of light signal with amount of positive clones present in cultivation.

Due to the multiple antibiotic resistance of *A. baylyi* strains, there was apparent lack of markers for transformation of these microbes. As was demonstrated in this study, luminescence hardly can be used as the marker for the purposes of accurate accounting of obtained clones. On the other hand, luminescence was perfect as the indicator of transformation dynamics in liquid cultivations, since even trace amount of luminescence produced in the very beginning of transformation was detectable providing quite high signal resolution in time.

At the same time, results displayed with conventional plating method, as was demonstrated, not always corresponded to the real breakdown of transformed and parent cells. For example, usage of 25 and 50 μg/ml CAM plates for selection of transformants resulted in significant difference in numbers of luminescent colonies for the same transformation mixture, coming from the death of transformed cells less resistant to antibiotic. In this aspect, the use of luminescence was advantageous since it reflected the full picture and covered all luminescent clones regardless of work of antibiotic resistance marker. Plating method, however, also had some benefits since it allowed identifying the fact of not only the target transformation but also let to see imperfections in the work of particular constructs in the form of the non-luminescent background.

During the experiments it was revealed that the efficient use of the system was compromised by production of luminescence background by *A. baylyi* ADP1 already con-

taining *luxABE* gene. Even mutants lacking 3383 gene produced approximately 10^4 luminescence counts, which was more than twice less than non-mutant clones and proved that fatty acyl-CoA reductase *acr1* only partially contributes to the substrate aldehyde production in *A. baylyi* ADP1 cells. Perhaps, the presence of *luxE* gene, which encodes one of three enzymes responsible for substrate production, can be the reason.

7.2 Following transformation in real time

However, in some cases conventional plating and luminescence methods can compensate disadvantages of each other, as was shown in current study. Such, developed system appeared to be inefficient for tracking the transformation of *A. baylyi* ADP1 with iKAN/pIX/*luxABE*/dCAM in real time. To my best knowledge, there is no apparent explanation to this phenomena in literature. After demonstration of the right structure of all constructs comprising the system and basing on the absence of luminescence signal in this experiment it was logical to conclude the fail of transformation. Nevertheless, conventional plating method demonstrated the presence of increasing over time amount of positive clones.

In contrast to this, new system worked perfectly for observing real time transformation of *A. baylyi* ADP1 with almost the same i/pIX/*luxABE* construct differing from the previous one only by resistance marker in cassette, while damaging of CAM^R gene was experimentally proved not to affect the real time light emission during the transformation. Plating, on the contrary, did not demonstrate any convincing level of target transformation and its match to the growth of luminescence. Therefore, in the mentioned experiment developed system proved the superiority over the conventional plating not only in terms of observation of transformation dynamics but also in terms of confirming successful transformation itself. Since, despite application of different approaches to the problem, the solution that would allow combining both features in the work of single i-construct containing luciferase genes was not found, two mentioned constructs were suggested for use depending on the purpose of experiment: either for tracking of signal increase in time or determining the transformation frequency based on positive colony number.

Taking into account the complexity of the events following the introduction of target DNA to the competent *A. baylyi* cells, it is possible to assert that time required for production of luminescence characterizes not only transformation and recombination events, but also gene expression, protein folding and transport as well as corresponding catalyzed reaction and availability of substrates and cofactors for them. If some of these steps suddenly stuck then the work of the whole system is ruined. Therefore, multiple factors should be taken into account during interpretation of results.

Since only two selective resistance markers are available for experiments with *A. baylyi* ADP1, trying different combinations of markers and cassettes can be the beneficial policy on the way towards improvement of system functionality. From already tested i/pIX/*luxABE*, iKAN/pIX/*luxABE*/dCAM and j/pIX/*luxCD*/dCAM the combinations

j-CAM and i-KAN appeared to be well-functioning for selection on plates, while combining of i-cassette and CAM^R led to fail in selection but allowed to follow luminescence. Therefore, it would be worth to construct and test other combinations, in particular, iCAM/pIX/*luxABE*/dCAM and jKAN/pIX/*luxCD*/dCAM. In addition to that, it is possible to suggest that problems with real time luminescence increase in experiments with iKAN/*luxABE*/dCAM could be somehow connected with change of membrane permeability for decanal in iKAN/*luxABE*-positive clones in liquid cultures. Then the exchange of *lux*-genes between constructs to obtain iKAN/pIX/*luxCD*/dCAM and j/pIX/*luxABE*/dCAM can potentially solve this problem, since decanal in this case would only be used for transformation with j-construct and it will be unnecessary to use it with i-construct, because *luxCD* genes would supposed to ensure the substrate production already in the cells.

Two-component system comprised by the tested versions of i- and j-constructs demonstrated the potential in identifying even low transformation rate events in experiments with an automatic luminescence reading mode, where bacterial growth and transformation happened slower as well as the corresponding luminescence increase. Since experiment was carried out automatically, because both constructs contained the same resistance marker, no data was obtained about the corresponding number of positive clones. However, I can suggest for these two constructs with similar resistance markers the way to differentiate between colonies with each of the cassettes separately and with both cassettes in genome. The only assumption for that is that all colonies present on the plates are transformed with cassette or its main *lux*-genes carrying part, as it happened in rare cases of random recombination. For this assumption to be fair i/pIX/*luxABE* construct used in experiment should be engineered to remove CAM^R in the plasmid backbone to get rid of the background colonies discussed in the section 5.1.1. Then it should be theoretically possible to identify clones with both cassettes on the basis of luminescence production. Further, the addition of decanal would help to identify the presence of only i/*luxABE*-cassette with subtraction of those clones luminescent without decanal. Finally, all non-luminescent clones could be considered containing only j/*luxCD*-cassette. In this way for simultaneous two-component transformation, it would be possible to count transformation frequencies for particular constructs and correlate them with the percent of double transformation. The results of such an accounting have to be quite reliable since all the indicators would be taken from the same plate.

For three-component system, in case it would successfully work, this advantage would be lost. The number of full *lux*-operon positive clones also would be revealed with the use of luminescence, and *luxA* and *luxBE* positive clones – with use of decanal. Since it is possible to operate only with two resistance markers, it would be real to count the number of clones only for cassette with unique marker by means of seeding it on the separate plate, while other two constructs would have similar resistance gene and, therefore, would be indistinguishable with simple plating. In addition to that, as was observed on practice, *A. baylyi* ADP1 transformants grow very slowly on plates containing both CAM and KAN antibiotics and colonies still appear after two days of surface cul-

tivation. Taking into account the fact of gradual loss of luminescence by colonies discussed in section 5.1.5, it would be unreliable to account for luminescent and non-luminescent clones after the time required for all clones to become visible. Moreover, from two-system transformation experiment it is clear that the probability of single cell transformation with two constructs at the time of *A. baylyi* competence was quite low and the change of the signal was negligible. Therefore, is seems obvious that three-cassettes transformation events are very unlikely for the current model and most probably won't be detected unless alternative more efficient cassettes with different flanking regions for knocking out of other theoretically not vital genes are developed.

7.3 Horizontal gene transfer

The developed system was successfully applied for studying the HGT in experiments with co-culturing of competent *A. baylyi* ADP1 T16 clone already containing iKAN/*luxABE* cassette with *E. coli* XL-1 Blue harboring j/pIX/*luxCD*/dCAM plasmid. It was demonstrated that *A. baylyi* during co-cultivation gained the missing *luxCD* part of operon and liquid culture began to produce luminescent signal. Since there are several mechanisms able to be suggested as the main, additional experiments were conducted to try to differentiate between them. These tests revealed that the majority of *lux*-operon positive clones resulted from simple uptake of DNA released from the dead cells. However, some positive clones were also present in co-culturing with *E. coli* cells but it is difficult to conclude reliably whether they appeared as a consequence of cell-to-cell communication or again just due to the traces of free DNA remained after the centrifugation or newly released from the dying *E. coli* cells in the process of cultivation.

Theoretically, the similar kind of system can be employed for studying the possibility of HGT between *A. baylyi* ADP1 or any other naturally competent bacteria and other species by means of engineering the plasmid vector for the broader host range and change of the flankings in cassettes.

Conclusions

This study has made an attempt to create the new system for tracking the natural transformation in the real time with use of luminescence production as a fast measure of transformation extent. The developed system presents the set of two vectors each containing the half of lux-operon, either luciferase genes or genes responsible for aldehyde substrate production.

It was suggested at the beginning that by revealing the interconnection between amount of produced luminescence and amount of positive transformants on plates it would be possible to receive the same correlation for any knock-out target, i.e. apply it for quantitative studying and characterization of natural transformation omitting the plating step and refusing of any additional markers apart from luminescence. Knock-out targets in the used constructs can be easily changed by simply changing the regions flanking the cassettes in vectors. The system was tested for this hypothesis with several combinations of knock-out genes and antibiotic resistance markers, revealing the significant divergence in function of each particular combination.

In contrast to conventional plating method, which is applicable to virtually any transformation with the only requirement of good selection marker, the developed system characteristics appeared to be strongly dependent on definite knock-out target. During the experiments it was shown that depending on these targets changed the transformation frequency, e.g. j-cassette gave the higher amount of transformants than i-cassette or SM100-cassette that did not work at all. In addition to that, antibiotic markers functioned in a different way for different cassettes transformed in the same bacteria, e.g. CAM^R gene resulted in almost none colonies on i-transformation plates despite the obvious presence of transformants in liquid culture evidenced by luminescence increase, while the same CAM^R gene worked perfect for j-transformation. Therefore, taking into consideration all the issues discussed above and in previous sections, it is possible to make the manifest conclusion that for every new knock-out target gene the system will work in a different way, i.e. it is not universal. Accordingly, the initial goal to correlate the light production with the amount of positive transformants quantitatively and calibrate the system for more or less universal application proved to be difficult.

Nevertheless, the developed system was successfully applied for trial experiments to study the HGT between *A. baylyi* ADP1 and *E. coli* XL-1 Blue and can be modified to explore this event between other bacteria by engineering the cassette carrying vector to the broad-host range vector.

Additionally, it will be beneficial to apply the system for further qualitative studying of influencing of different factors on natural competence and transformation of *A. baylyi*

or, virtually, any other competent recombination-proficient microbe. This will not only allow to reduce significantly the time of experiments from days typically needed for surface cultivation to hours required for luminescence detection right in the transformation mixture, but also will make the whole experiment less laborious.

Moreover, the system demonstrated the promising results in observing and confirming two-component simultaneous transformation in real time with potential to be further developed for this application. Furthermore, some modifications to the system discussed previously can finally lead to success of experiments, aimed on comparison of natural plasmid transformation and natural transformation with subsequent recombination, described in section 5.2.3. As both, conventional method and luminescence detection methods were used in this study to design, characterize and test the system and overall experiments were quite laborious and time-consuming, there was unfortunately the luck of time to examine all the ideas suggested in the previous sections for improvement of the resulted system.

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APPENDIX 1: SCRIPT FOR CALCULATION OF DILUTIONS

```
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    <title>Co-culturing</title>
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        address { display: none; }
        .output td { padding: 3px; }
        .warning { background: #fcc; }
        span { color: #090; font-weight: bold; }
        td span { display: block; text-align: center; }
        input { font-family: monospace; text-align: center; }
    </style>
    <style media="print">
        address { display: inline; }
        var { display: none; }
    </style>
</head>
<body>
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        </tr>
        <tr>
            <td>E.coli j NEW:</td>
            <td><input id="D1"></td>
        </tr>
        <tr>
            <td>E.coli j OLD:</td>
            <td><input id="D2"></td>
        </tr>
        <tr>
            <td>E.coli NEW:</td>
            <td><input id="D3"></td>
        </tr>
        <tr>
            <td>E.coli OLD:</td>
            <td><input id="D4"></td>
        </tr>
    </table>

    <h3>Output</h3>
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            <th></th>
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            <th>LB</th>
        </tr>
        <tr>
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            <td><span id="VDM1"></span></td>
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        <tr>
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        </tr>
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        <tr>
            <td>E.coli OLD</td>
            <td><span id="VD4"></span></td>
            <td><span id="VDM4"></span></td>
        </tr>
    </table>
    <p>V ADP1 = <span id="VX"></span></p>

    <var><a href="javascript:window.print();">Print</a></var>
    <address>&copy; 2013 Elena &amp;amp; Alexander Lokhman</address>

    <script>
        var vmax  = 4.7,
            input = document.getElementsByTagName("input"),
            _VX   = document.getElementById("VX"),
            _VD1  = document.getElementById("VD1"),
            _VD2  = document.getElementById("VD2"),
            _VD3  = document.getElementById("VD3"),
            _VD4  = document.getElementById("VD4"),
            _VDM1 = document.getElementById("VDM1"),
            _VDM2 = document.getElementById("VDM2"),
            _VDM3 = document.getElementById("VDM3"),
            _VDM4 = document.getElementById("VDM4"),
            isNumber = function(n) {
                return !isNaN(parseFloat(n)) && isFinite(n);
            },
            update = function(e, n) {
                e.innerHTML = n === n ? n.toFixed(3) : "[ x ]";
            };

        for (var i = 0, len = input.length; i < len; i++) {
            input[i].addEventListener("keyup", function() {
                var x  = parseFloat(input[0].value),
                    d1 = parseFloat(input[1].value),
                    d2 = parseFloat(input[2].value),
                    d3 = parseFloat(input[3].value),
                    d4 = parseFloat(input[4].value),
                    dmin = Math.min(d1, d2, d3, d4),
                    vx = vmax * dmin / (x + dmin),
                    vxx = vx * x,
                    vd1 = vxx / d1,
                    vd2 = vxx / d2,
                    vd3 = vxx / d3,
```

```
                    vd4 = vxx / d4,
                    vdmin = vxx / dmin;

                update(_VX, vx);
                update(_VD1, vd1);
                update(_VD2, vd2);
                update(_VD3, vd3);
                update(_VD4, vd4);
                update(_VDM1, vdmin - vd1);
                update(_VDM2, vdmin - vd2);
                update(_VDM3, vdmin - vd3);
                update(_VDM4, vdmin - vd4);

                this.className = isNumber(this.value)?"":"warning";
            }, false);
        }

        input[0].focus();
    </script>
</body>
</html>
```